The effect of soil aggregate size and pH on nitrous oxide emissions, ammonia oxidising communities and DCD effectiveness in a grazed pasture soil

A thesis submitted in partial fulfilment of the requirements for the Degree of Master of Science

at Lincoln University by Aimee Robinson

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by

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Agricultural soils have been identified as the main anthropogenic source of nitrous oxide (N_2O) emissions. N_2O is produced microbially through the processes of denitrification and nitrification as part of the nitrogen cycle. Soil properties can amplify N_2O emissions by creating a favourable environment for N_2O production or by altering microbial pathways. However, the impacts of soil properties such as aggregate size and soil pH on N_2O emissions have yet to be fully understood. Thus the objectives of this research were to: 1) quantify N_2O emissions from a grazed pasture soil with different soil aggregate sizes and soil pH; 2) determine the effectiveness of the nitrification inhibitor DCD in reducing N_2O emissions from a soil over a range of soil aggregate sizes and pH values; and 3) determine changes in ammonia oxidising bacteria (AOB) and ammonia oxidising archaea (AOA) abundance as affected by soil aggregate size and pH.

An incubation trial and field trial was carried out to assess the effects of soil aggregate size and soil pH, respectively, on N_2O emissions, ammonia oxidising communities and DCD effectiveness.

For the incubation trial a Temuka clay loam soil was sieved to produce three aggregate sizes: large (4-5.6 mm), medium (2-4 mm) and small (1-2 mm). These aggregate sized soils were

incubated at 10˚C for 397 days in gas sampling jars and soil sampling tubes. Temporally, N_2O emissions were different, with higher peak N_2O emissions seen in the large and medium aggregates. However, high N_2O emissions after day 66 from the small aggregates meant that total emissions were not significantly different between aggregate sizes. Increased N_2O emissions after day 66 from the small aggregates are thought to be caused by greater aggregate instability causing aggregate disruption and a release of previously unavailable carbon. Ammonia oxidising communities were not affected by aggregate size, and DCD was effective in all aggregate size treatments, reducing N_2O emissions by an average of 79%.

The field trial was established at Lincoln University in a Temuka clay loam soil. The soil pH was altered using HCl for the 'acidic' pH plots (pH < 5) and CaO/NaOH in the 'basic' pH plots (pH > 6). Water was used for the control pH plot and refered to as the 'native' pH soil. Total N_2O emissons were significanly higher in the acidic pH soil compared to the native and basic pH soils. This is hypothesised to have been caused by inhibition of the N_2O-reductase enzyme in the denitrification pathway. Ammonia oxidising microbes were affected by soil pH with AOB *amoA* gene copy numbers increasing in the basic pH soil and AOA *amoA* gene abundance increasing in the acidic pH soil. The addition of urine enhanced AOB growth and inhibited AOA growth. This supports the previous reseach that AOA prefer low nutrient, low pH environments whilst AOB prefer high N concentrated soil. DCD was most effective in the acidic pH soil reducing total N_2O emissions by 64%.

Keywords: Nitrous oxide, soil pH, soil aggregate size, nitrate, ammonium, ammonia oxidising bacteria, ammonia oxidising archaea, urine, nitrification inhibitor, DCD.

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Chapter 1
Introduction

Greenhouse gases (GHG's) naturally provide radiative forcing in the earth's atmosphere allowing the climate to warm (Intergovernmental Panel on Climate Change 2007a). Anthropogenic influences have intensified this greenhouse effect through the release of GHG's such as carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O). The alteration of atmospheric gas composition has led to climatic changes through global warming (Intergovernmental Panel on Climate Change 2007b). The main sources of anthropogenic GHG's are transport, energy supply, industry, forestry and agriculture (Intergovernmental Panel on Climate Change 2007b). Worldwide growth and expansion of these sectors has significantly increased the concentration of the aforementioned GHG's in the atmosphere in the last 100 years.

In New Zealand, agricultural emissions make up a major part of the GHG profile (Ministry for the Environment 2011). In 2009, agricultural emissions equated to 46.5% of the total GHG emissions. This high agricultural contribution is much more than the global average of 10% for developed countries (Ministry for the Environment 2011). Agriculture in New Zealand is a significant part of the economy and contributes to over half of the annual merchandise exports (Ministry for the Environment 2009). The expansion and intensification of the agriculture sector in New Zealand has resulted from the rise in worldwide demands for meat and dairy products as well as increasing prices. The reduction of GHG's is important for New Zealand's commitment to the Kyoto protocol, as agriculture is our main source of GHGs, it is therefore imperative that this sector is the focus for GHG research and mitigation.

In 2009 N_2O made up 17.0% of New Zealand's total GHG emissions and agriculture produced 96% of these emissions (Ministry for the Environment 2011). This high production of N_2O from agriculture is a function of New Zealand's year round pastoral based farming, from which the majority of emissions are caused by the deposition of animal excreta onto soils during grazing (de Klein et al. 2006). N_2O is produced as part of the microbial driven nitrogen (N) cycle, through the processes of nitrification and denitrification. The first step in nitrification, ammonia oxidisation, is the rate limiting step as it provides the substrate for N_2O formation. Ammonia oxidising bacteria (AOB) and archaea (AOA) carry out this process, with AOB as the predominant organism responsible. However, in low nutrient and/or acidic

soils AOA can become more dominant (Leininger et al. 2006; Di et al. 2009). The response of AOA and AOB to soil pH changes, and how these microbial community changes could impact N_2O emissions, is not well understood or documented in the literature.

It has been demonstrated that N_2O emissions can be enhanced due to environmental conditions, such as high rainfall and temperatures, agricultural management, and soil conditions such as soil moisture, aeration, texture, pH, and the concentration of carbon (C), N, ammonium (NH_4^+) and nitrate (NO_3^-) (de Klein et al. 2001). Fewer studies demonstrate the impact of aggregate size on N_2O emissions. Soil aggregate size variation is a soil property which is thought to alter emissions by creating differing amounts of anaerobic "hotspots" for N_2O production. However, there does not appear to be agreement on the aggregate size at which anaerobic zones will be enhanced (Sexstone et al. 1985; Uchida et al. 2008; Diba et al. 2011), and no studies determine the effects aggregate size will have on ammonia oxidising communities.

Nitrification inhibitor (dicyandiamide, DCD) application has been shown to be a method to reduce N_2O emissions (and NO_3^- leaching) from agricultural soils (de Klein et al. 2001; Di & Cameron 2002). Nitrification inhibitors work by inhibiting the enzyme responsible for the conversion of NH_4^+ to NO_3^- (ammonia mono-oxygenase). Studies have previously shown effectiveness of DCD under varying soil conditions (Di & Cameron 2004, 2006; Singh et al. 2008; Luo et al. 2010b; de Klein et al. 2011), however few have determined the effectiveness with varying aggregate size or soil pH.

A review of the literature has identified significant gaps in knowledge and understanding about the effects of soil pH or aggregate size on N_2O emissions from grazed pasture soils and whether these emissions can be reduced through the application of a nitrification inhibitor. There is also lack of knowledge and understanding about the effects of soil pH and aggregate size on AOB and AOA populations in soil.

1.1 Aims and Objectives

The aim of this study is to gain a fundamental understanding of the effect of soil aggregate size and pH on N_2O emissions from an agricultural soil, as well as the effectiveness of DCD in reducing N_2O emissions in these soil conditions. The ultimate aim is to develop improved mitigation technologies for reducing N_2O emissions from pastoral agriculture. In addition, this study will also increase our knowledge on how AOA and AOB contribute to N_2O emissions, and how soil aggregate size and pH affects their population abundance.

These goals will be realised by achieving the following objectives:

1. To quantify nitrous oxide emissions from an agricultural soil with different soil aggregate size and soil pH;

2. To determine the effectiveness of the nitrification inhibitor DCD in reducing nitrous oxide emissions from soil over a range of soil aggregate sizes and pH values;

3. To determine changes in AOA and AOB abundance in agricultural soils with changes in soil aggregate size and pH.

1.2 Hypotheses

It is hypothesised that:

1. Nitrous oxide emissions will be significantly affected by aggregate size and pH, with N_2O emissions being higher in the larger aggregate sized soil and the lower pH treatment and;

2. The nitrification inhibitor DCD will continue to be equally effective in reducing N_2O emissions regardless of soil aggregate size and be more effective at a low soil pH and;

3. Soil aggregate size and pH will significantly affect the abundance of ammonia oxidising bacteria (AOB) and ammonia oxidising archaea (AOA), with AOA becoming more dominant in the low pH treatments.

Chapter 2
Review of the Literature

2.1 Introduction

Nitrous oxide (N_2O) is an important greenhouse gas (GHG) with an atmospheric lifetime of 114 years (Intergovernmental Panel on Climate Change 2007a). Although N_2O emissions are low in proportion to other greenhouse gases, N_2O has almost 300 times greater global warming potential compared to carbon dioxide. This is due to its long lifetime and high radiative forcing. Furthermore, N_2O also has the ability to destroy ozone, a stratospheric gas important for keeping out harmful ultraviolet rays (Intergovernmental Panel on Climate Change 2007a).

The majority of N_2O is produced biologically through the respiratory processes of nitrification and denitrification as part of the nitrogen (N) cycle in soil (Thomson et al. 2012). Naturally the N cycle is limited by the availability of ammonia (NH_3) produced by N fixing bacteria and the breakdown of organic matter. However, the development of the Haber-Bosch process allowed the reduction of atmospheric di-nitrogen (N_2) to NH_3 to occur anthropogenically (Thomson et al. 2012). This allowed agriculture to intensify through the use of synthetic N based fertilisers, increasing the amount of bioavailable and reactive N in the soil leading to enhanced N_2O emissions. The concentration of atmospheric N_2O has been increasing at an annual rate of 0.2-0.3% per year (Watson et al. 1992) and the total atmospheric concentration has increased by 20% in the last century (Thomson et al. 2012).

In New Zealand, N_2O emissions account for 17% of our total greenhouse gases, of which 96% is from agriculture (Ministry for the Environment 2011). Agriculture in New Zealand is distinctive with year round pastoral farming (de Klein et al. 2003). This unique farming practice is facilitated by New Zealand's temperate climate and wealth of agricultural land (Ministry for the Environment 2011). Clover based pasture provides a natural N source, decreasing the reliance and use of fertilisers. As a result, over 80% of the direct and indirect emissions are caused by the deposition of animal excreta during grazing (de Klein et al. 2006), either directly as deposited, through volatilisation, or by leached excreta N (de Klein et al. 2003). In comparison, annual fertiliser and effluent only contribute 14% and 3% of agricultural emissions respectively (de Klein et al. 2006).

2.2 Biological production of Nitrous Oxide (N_2O)

2.2.1 Nitrification

Autotrophic nitrification is a biological process carried out by nitrifying microbes such as *Nitrosomonas* and *Nitrosospira* which convert ammonia (NH_3) to nitrate (NO_3^-), and *Nitrobacter* species which convert nitrite (NO_2^-) to nitrate (NO_3^-) (Reijnan 2002). Nitrification is the precursor to denitrification, providing the substrate for denitrifiers to produce N_2O and N_2. Thus, increases in ammonia oxidising communities may enhance N_2O emissions from soil. Furthermore, nitrification itself can produce N_2O through a separate reductive side reaction in oxygen (O_2) limiting conditions, where ammonia oxidisers use NO_2^- as the electron acceptor instead of O_2 (Sherlock et al. 1992). This process is known as nitrifier denitrification (Figure 2.2). Nitrifier denitrification can significantly contribute to N_2O emissions in soils and the process is the same, enzymatically, as denitrification (Baggs 2011). This process has been thought to be a mechanism to avoid the potentially toxic accumulation of NO_2^- in the soil.

Ammonia oxidising bacteria (AOB) are not the only organisms involved in nitrification. In certain environmental conditions archaea can drive the N cycle as they can perform both dissimilatory and assimilatory processes (Cabello et al. 2009). Ammonia oxidising archaea (AOA) produce the same enzyme as AOB, ammonia monoxygenase (*amo*A). However the gene encoded for it is unique to the archaea. Leinginer et al. (2006) states that archaea could represent the most abundant ammonia oxidising organism in the soil ecosystem. Archaea are important as they provide another source of N_2O through the same processes as AOB. Archaea's abundance in soil relative to bacteria is high, but these numbers are not reflective of their role in nitrification (Di et al. 2009) and it is proposed that AOA may be more important in low nutrient and low pH environments (Erguder et al. 2009). Species of archaea are hard to cultivate in the laboratory hence there is limited understanding of their physiological characteristics (Killham & Prosser 2007). This means that their role in soil, and in N_2O production, is mainly hypothesised.

2.2.2 Denitrification

Nitrous oxide is produced in the soil as an intermediate product of microbial nitrification and denitrification as part of the N cycle (Figure 2.1) (Delwiche 1981). Denitrification is the reduction of NO_3^- or NO_2^- to gaseous products, mainly N_2 (Reijnan 2002; Spanning et al.

2007) with N_2O as an obligatory intermediate (Eckard et al. 2010). Denitrification is a specific respiratory process which is carried out in anaerobic conditions where bacteria replace O_2 with NO_3^- or NO_2^- as the electron acceptor for the oxidation of organic matter N (Delwiche 1981; Sherlock et al. 1992; Spanning et al. 2007). This results in energy and the release of N_2 or N_2O. Denitrifying bacteria will only use NO_3^- when O_2 is otherwise unavailable due to the low efficiency of NO_3^- as an electron acceptor (Robertson & Groffman 2007). This occurs in soil when the water pores reach saturation and the diffusion of O_2 is low causing localised anaerobic conditions, usually after rainfall events.

$$\mathbf{NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2}$$

(1) (2) (3) (4)

Figure 2.1 The denitrification pathway which must have enzymes nitrate reductases (1), nitrite reductases (2), nitric oxide reductases (3), and nitrous oxide reductases (4) present for the pathway to be complete (Reijnan, 2003; Ingraham, 1981).

Specific complex multisite metalloenzymes (Spanning et al. 2007) produced by different bacteria catalyse each step in the denitrification pathway (Figure 2.2). Not all denitrifying organisms (e.g. heterotrophs) (Robertson & Groffman 2007) are able to synthesis all of the enzymes required for the complete reduction, however true denitrifiers are those which possess the entire pathway (e.g. *Pseudomonas* species) (Coyne 1999). The combination of denitrifying organisms in the soil can cause either complete or incomplete reduction of NO_3^- to gaseous N_2. Hence, incomplete or partial denitrification can occur when an intermediate of the pathway is not present; environmental conditions are unfavourable or there is a lack of those organisms which are genetically capable of producing the complete array of N oxide reductases (Ingraham 1981). It is the occurrence of incomplete denitrification which causes high production of N_2O in soil. The ability to denitrify is phylogenetically diverse and can be undertaken by microbes belonging to different functional groups. For example ammonia oxidizing bacteria (AOB) are also able to denitrify. This is referred to as nitrifier denitrification (Thomson et al. 2012).

Nitrification and denitrification can occur at the same time when conditions for both processes are favourable (Wrage et al. 2001). This process, called coupled nitrification-denitrification, is when the NO_2^- or NO_3^- produced by nitrifiers is used by denitrifiers to produce either N_2O or N_2. For example, nitrification could occur in the aerobic surface layers, whereas

denitrification would be confined to the anaerobic subsurface or waterlogged layers or the centre of larger aggregates. Khdyer and Cho (1983) found coupled nitrification-denitrification produced N_2O at the interface of aerobic and anaerobic zones where the N_2O produced could diffuse to the soil surface.

Some non-denitrifying fungi and bacteria can also yield N_2O, biologically producing it as a transient by-product during the process of dissimilatory reduction of NO_3^- to ammonium (NH_4^+) (Sherlock et al. 1992). This usually occurs in environments where prolonged anaerobic periods occur, such as in rice paddy fields or sediments.

Non-biological processes such as chemo-denitrification can also cause N_2O production in soils (Sherlock et al. 1992). This is where nitric acid (HNO_3) is chemically decomposed to NO_3^-. This accumulated NO_3^- can then be denitrified by the denitrification process to produce N_2O. Chemo-denitrification occurs in soils where the pH is low. In contrast in neutral to alkaline soils, biological processes are usually responsible for N_2O production. In New Zealand the chemo-denitrification process is of minor importance as it is the addition of animal excreta which produces the major proportion of N_2O (Oenema et al. 1997). Furthermore N_2O production is mainly affected by soil moisture, hence at or above field capacity, the main mechanism for N_2O production will be denitrification (Smith et al. 1998).

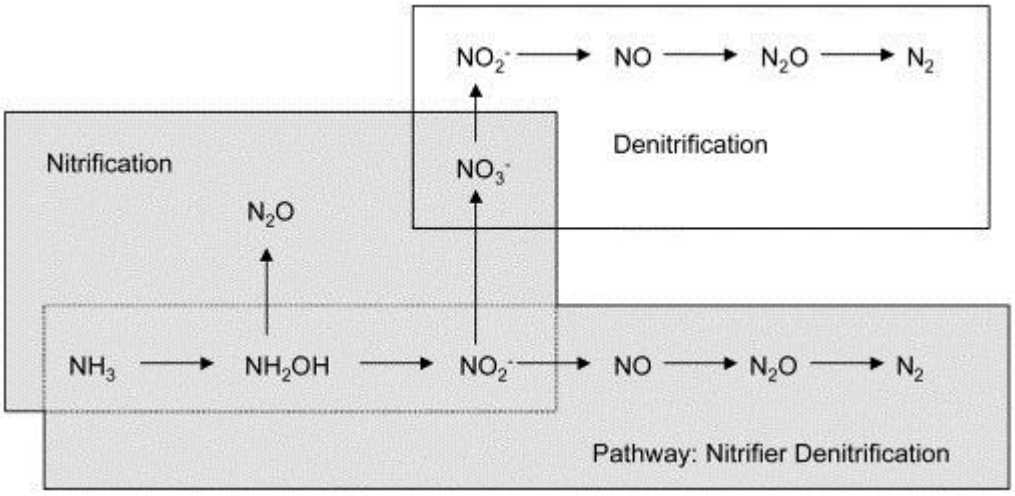

Figure 2.2 Transformations of mineral nitrogen in soil (Wrage et al. 2001).

2.3 Soil attributes affecting N_2O emissions and microbial communities

Nitrous oxide emissions from soil relies on the complex interaction between soil properties, climatic factors, and agricultural practices (Figure 2.3) (Choudhary et al. 2002). The main factors in the soil are soil moisture and seasonality, temperature, texture, pH, and the concentration and availability of organic matter, NO_3^- and NH_4^+ (de Klein et al. 2001). Nitrous oxide emissions may not always strongly correlate with these factors, but it is their interaction which can either enhance or diminish N_2O emissions.

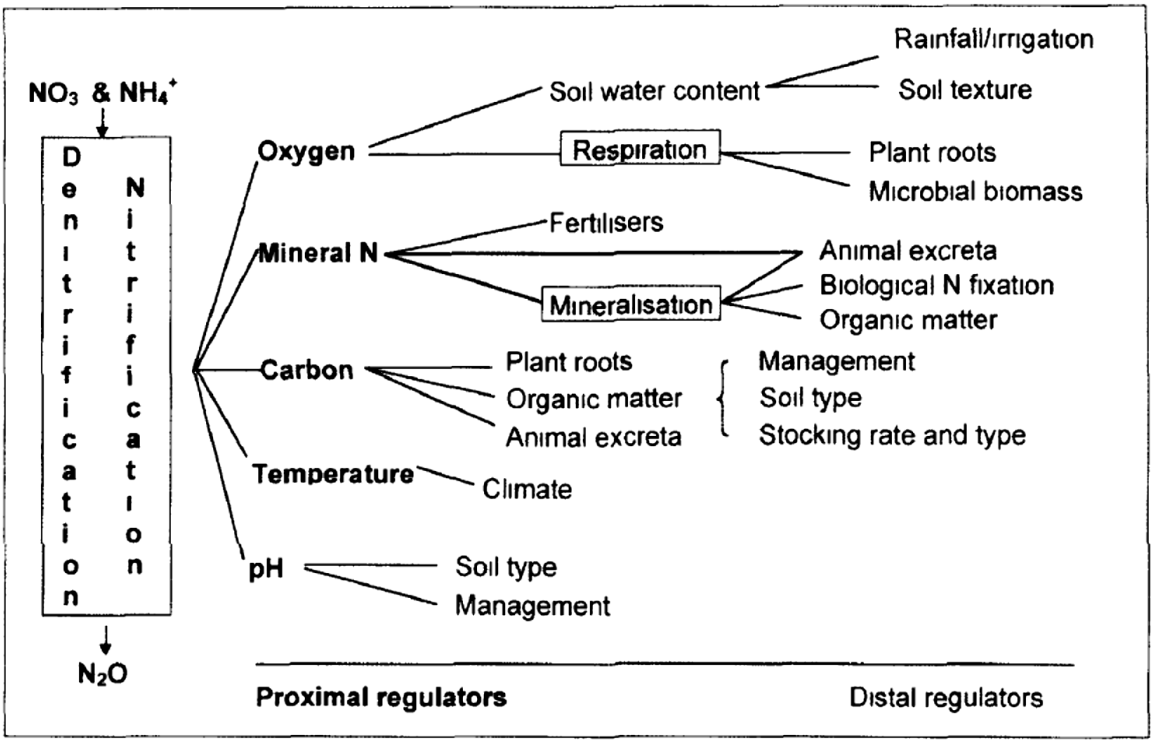

Figure 2.3 Systematic diagram of the proximal and distal regulators which effect nitrous oxide emissions in agricultural soils, boxes represent biological processes (de Klein et al., 2001).

2.3.1 Soil moisture and aeration

Soil water content affects N_2O emissions by providing localised anaerobic conditions for denitrification to occur. Moisture conditions which favour N_2O high emissions are alternating wetting and drying cycles (Sherlock et al. 1992). This is because both autotrophic nitrification and denitrification occur, however there is not enough time for the N_2O to be

reduced further to N_2 as the soil dries. Smith et al. (1998) found that N_2O emissions decreased in a clay loam soil when the water filled pore space (WFPS) was higher than 90%. In contrast, when the WFPS was 80%, emissions were enhanced. This was caused by the transformation of N_2O to N_2 in complete anaerobic conditions. Furthermore, in soils with a lower WFPS, incomplete denitrification occurs and more N_2O is produced. This is because the gene encoded for N_2O-reductase, which converts N_2O to N_2, is regulated by the presence of O_2 rather than N_2O concentrations (Thomson et al. 2012). Hence when the O_2 concentration in the soil decreases, as the soil dries, more N_2O-reductase is produced completing the denitrification pathway. This explains why a higher proportion of N_2O:N_2 is produced in anaerobic conditions.

Production of N_2O by autotrophic nitrification can also be enhanced following additions of water to soil. This is caused by the stimulation of the microbial community, with increases in readily available water (Sherlock et al. 1992). Maag and Vinther (1996) found that the percentage of N_2O-N produced by nitrification increased with increasing soil moisture contents. Bateman and Baggs (2005) found that autotrophic nitrification was the predominant process producing N_2O from treatments with a WFPS of 35% to 60%. A WFPS less than 60% is optimal for nitrification because there is no restriction on the diffusion of substrates or O_2. However, the higher N_2O emissions seen in the 60% WFPS could have been a combination of autotrophic nitrification and denitrifier nitrification as short term O_2 limitation could have occurred (Bateman & Baggs 2005).

Seasonal variation causes changes in WFPS which directly alters the N_2O emissions from soil. During wetter months, in autumn and winter, the WFPS increases causing greater denitrification rates and higher N_2O emissions. In grazed pastures 85% of the N_2O emissions occur when the WFPS exceeds 50% suggesting that during the autumn/winter months, when high WFPS is common, a disproportionally higher amount of the N_2O emissions will occur (de Klein et al. 2006). Luo et al. (2007) suggests avoiding fertiliser applications during wet winter and spring conditions could decrease N_2O emissions from pastoral soils. However, in summer, high temperatures can cause increased N_2O emissions, especially after a rainfall event (Saggar et al. 2007). This is caused by the stimulation of microbiological activity in the moist, warm conditions (Sherlock et al. 1992). Sagger et al. (2007) found N_2O emissions were five to ten times higher in summer than winter during a rainfall event followed by grazing. Similarly, Rafique et al. (2011) found that elevated N_2O emissions coincided with high WFPS, surface soil temperature and N application events. In contrast, Smith et al.

(1998) found that even with higher temperatures during summer, N_2O production was often low because of drier conditions. This demonstrates the importance of soil moisture when determining seasonal effects on N_2O emissions.

2.3.2 Temperature

At high soil temperatures, denitrification and autotrophic nitrification are enhanced (Sherlock et al. 1992) with the optimal temperature being 30^0C. This temperature is rarely reached in New Zealand soils, so under field conditions N_2O emission rates follow a diurnal pattern, which closely follows the day/night temperature fluctuations. These changes are more extreme at the soil surface and decrease with depth. Smith et al. (1998) found that when N_2O peaks coincided with temperature peaks, the N_2O production must have been in the uppermost part of the profile, compared to a seven hour lag peak which demonstrated N_2O production at lower depths.

Cooler temperatures result in lower microbial activity, increased N_2O solubility and slower gaseous diffusions causing decreased N_2O emissions (Sherlock et al. 1992). However, below 15^0C incomplete denitrification will produce higher N_2O emissions due to lower proportions of N_2O being converted to N_2. Keeney et al. (1979) concluded that even though the rate of denitrification was low at temperatures lower than 15^0C, the amount of N_2O emitted could be equivalent to the amount evolved at 25^0C due to incomplete denitrification. Maag & Vinther (1996) found that N_2O produced by nitrification was 3 times higher at 5^0C compared to 20^0C. This is caused by the accumulation of NO_3^- at low temperatures which leads to nitrifiers increasing their contribution to N_2O emissions as O_2 concentration decreases through respiratory use. Rafique et al. (2011) found that N_2O emissions were 5 times lower in soils with temperatures below 5^0C than 17^0C (Figure 2.4). Above 17^0C the relationship was lost and it was presumed other factors such as moisture content and N availability were more controlling (Rafique et al. 2011).

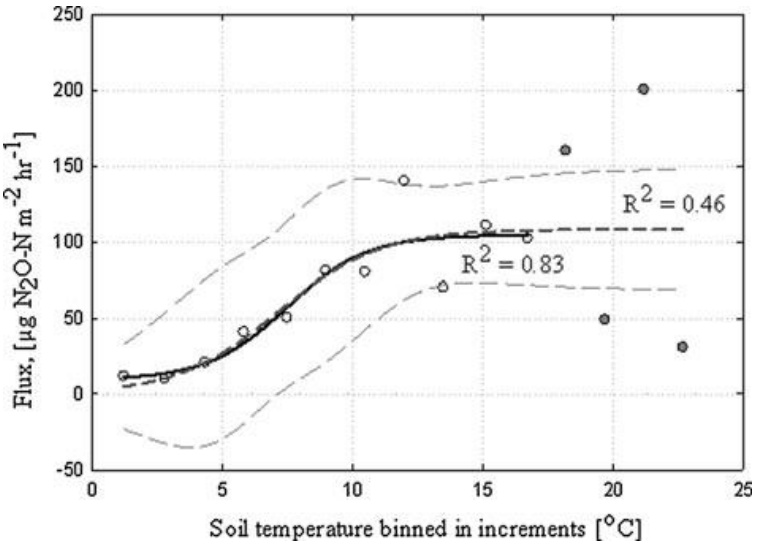

Figure 2.4 Nitrous oxide flux increases linearly with temperatures between 5^{0}C and 10^{0}C, above this temperature the relationship is lost (Rafique et al. 2011).

2.3.3 Soil texture

Free draining soils such as sandy soils have less N_2O emissions than those with poor or imperfect drainage such as clay soils (Luo et al. 2010a). Wodarczyk et al. (2011) found that soils formed from silt had higher N_2O efflux than those formed from sand. They explained that this was caused by the differences in pore size distribution and hence WFPS. Luo et al. (2010a) states that poorly drained soils can emit up to five times the amount of emissions compared to free draining soils. This is caused by a higher proportion of localised anaerobic conditions which cause higher denitrification rates. In contrast, Rafique et al. (2011) found that free draining podzols produced higher N_2O emissions than poor draining gley soils (Figure 2.5). This was due to the higher porosity seen in the podzols which enhanced nitrification. Furthermore, the gley soils were frequently waterlogged, leading to very high WFPS and almost completely anaerobic conditions (Rafique et al. 2011), reducing nitrification and probably leading to a more complete denitrification pathway.

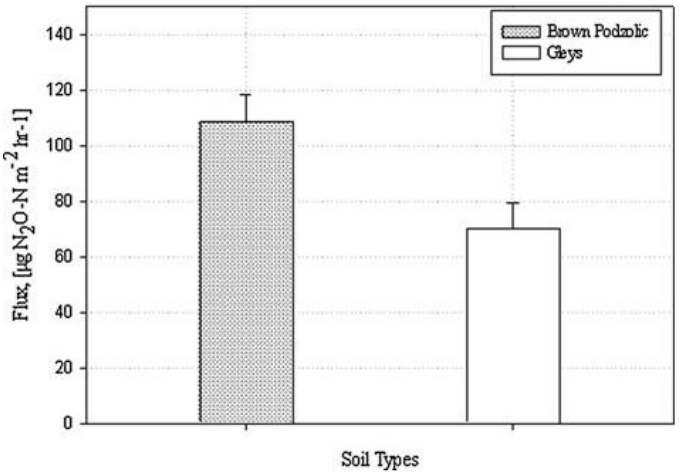

Figure 2.5 Lower N_2O fluxes are seen in gley soils (Rafique et al., 2011).

2.3.4 Soil aggregate size

Soil aggregate sizes can alter N_2O emissions by altering gas diffusion, aeration, porosity and thus WFPS. It is hypothesised that larger aggregates create localised anaerobic 'hotspots' within the soil aggregate where denitrification rates are higher than the surrounding soil. These hotspots are created when the microbial O_2 consumption is greater than the diffusive supply, with larger aggregates having lower O_2 diffusion (Figure 2.6) (Sexstone et al. 1985). Furthermore, larger aggregates can hold more water inside the micro pores further lowering aggregate O_2 concentration (Diba et al. 2011). Greenwood (1975) predicted that only if the aggregate radius exceeded 9mm that an anaerobic zone would be present. In contrast, Sexstone et al. (1985) found anaerobic zones in aggregates down to 4mm. However, not all of the aggregates with anaerobic zones denitrified, and denitrification rates were not correlated with the size of the anaerobic zone. Sexstone et al. (1985) concluded that factors other than anaerobic volume contributed to denitrification rates. Diba et al. (2011) found in a volcanic ash soil that larger aggregates produced more N_2O than smaller aggregates when treated with fertiliser and manure. In an anaerobic incubation study, Drury et al. (2004) found that N_2O emissions from denitrification increased with increasing intact aggregate size. Drury et al. (2004) and Diba et al. (2011) believe this is caused by the higher amounts of NH_4^+ and NO_3^- in the larger aggregates. Khalil et al. (2005) also found higher N_2O production from larger aggregates. Renault and Stengel (1994) found that small aggregates only become anaerobic when saturated, however larger unsaturated aggregates tend to have a constant anaerobic centre

The compaction of the aggregates can have significant effects on the N_2O emissions, as smaller aggregates can pack tighter than larger aggregates. Uchida et al. (2008) found that smaller aggregates (0-1mm) produced higher N_2O emissions than larger aggregates when applied with bovine urine. Furthermore, aggregates >5.6mm only produced N_2O after significant compaction as higher aeration between the soil aggregates lowered denitrification rates.

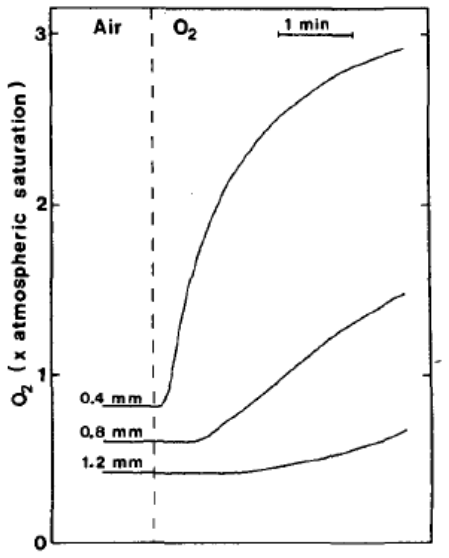

Figure 2.6 Oxygen diffusion in different aggregate sizes with an abrupt shift (at dashed line) in the atmosphere surround the aggregate from water saturated air to 100% oxygen (Sexstone et al., 1985).

2.3.5 Soil pH

Denitrification rates are affected by soil pH, with rates increasing at a higher pH (Rolston 1981). The optimum pH for denitrifying organisms is between pH 7 and 8 (Rolston 1981; Sherlock et al. 1992), with low denitrification rates in acidic conditions (Simek et al. 2002). In soils where long term pH changes have occurred, the bacterial community can shift towards those species which tolerate low pH's (Parkin et al. 1985). Liu et al. (2010) describes pH as a stable soil characteristic which provides a long term selective pressure which makes it a master variable in determining bacterial community compositions. Simek et al. (2002) found that when altering the soil pH, the denitrifying enzyme activity was highest in the soil pH closest to the natural pH of the soil. This indicates that the soil denitrifier community has adapted to the natural soil pH, thus changing the denitrifier's pH optima.

Although denitrification rates can decrease in a low soil pH, the ratio of N_2O:N_2 increases (Wijler & Delwiche 1954). At a high pH complete denitrification is favoured, consequently producing more N_2 than N_2O; whereas at a low pH, N_2O production is favoured (Wijler & Delwiche 1954). This is independent of the bacterial communities present (Simek & Cooper 2002) and is thought to be caused by the inhibition of N_2O-reductase at a lower pH (Fillery 1983). Weislien et al. (2009) found a strong negative correlation between N_2O emissions and pH in forested organic soils (Figure 2.7), with almost 5 times higher N_2O emissions from plots with a pH 3.7 compared to pH 5.8. Clough et al. (2004) found that N_2O emissions were higher in a soil pH less than 5.9 at field capacity. However when the soil was saturated the emissions were lowest in the pH 4.7 soil, highlighting the importance of soil moisture effects on N_2O emissions.

In lab studies of denitrifiers, Thomsen et al. (1994) found that *Paracoccus denitrificans* at a pH of 5.5 produced intermediates of denitrification (NO_2^-, N_2O). In contrast, at pH 8.5, the NO_3^- was converted completely to N_2 with only low concentrations of intermediates. They state that in a low pH the inhibitory effect on N_2O reduction was greater than the inhibitory effect on NO_2^- reduction which caused N_2O to accumulate. Bakken et al. (2012) found that *P. denitrificans* at pH 6 emitted nearly 100% of NO_3^- as N_2O. This was caused by an interference with the production of N_2O-reductase rather than the narrow pH maxima of the enzyme (Thomsen et al. 1994).

Like denitrifiers, nitrifiers are also sensitive to soil pH. This is important as nitrifiers supply the substrate for denitrification and thus N2O production. The optimum pH for nitrifiers, is 8 – 8.5, but can occur in a pH down to 6.5 (Shammas 1986). However, in acidic conditions, less than pH 5.5, nitrification rates, specifically NH_3 oxidation, are significantly lowered (de Boer & Kowalchuk 2001). This is caused by the decrease in NH_3 availability due to the protonation of NH_3 to NH_4^+ at a low pH. de Boer & Lannbroek (1989) found increased nitrification rates in acidic conditions with the addition of urea. Urea provided NH_3 for the enzyme ammonia monoxygenase (*amo*A) and reduced the need for the energy-dependant transport of NH_4^+ into the bacterial cell (Nicol et al. 2008).

In acidic soils nitrification still occurs, and it is thought that it may result from the selection of acidophilic ammonia oxidizers which have the required characteristics to adapt to a low pH environment (Nicol et al. 2008). Recent studies have hypothesised that archaeal communities may dominate ammonia oxidation in low pH soils due to their adaption to extreme

environments (Erguder et al. 2009; Di et al. 2010b). Nicol et al. (2008) found in long term pH plots that bacterial *amo*A decreased with acidity, whereas archaeal *amo*A increased. Zhang et al. (2011) state that AOA and AOB occupy different niches in acidic soils, with ammonia oxidation driven by AOA rather than AOB. The role of AOA in nitrification is yet to be fully understood and they could dominate N cycling and hence N_2O production in some low pH, low nutrient soils (Erguder et al. 2009; Di et al. 2010b).

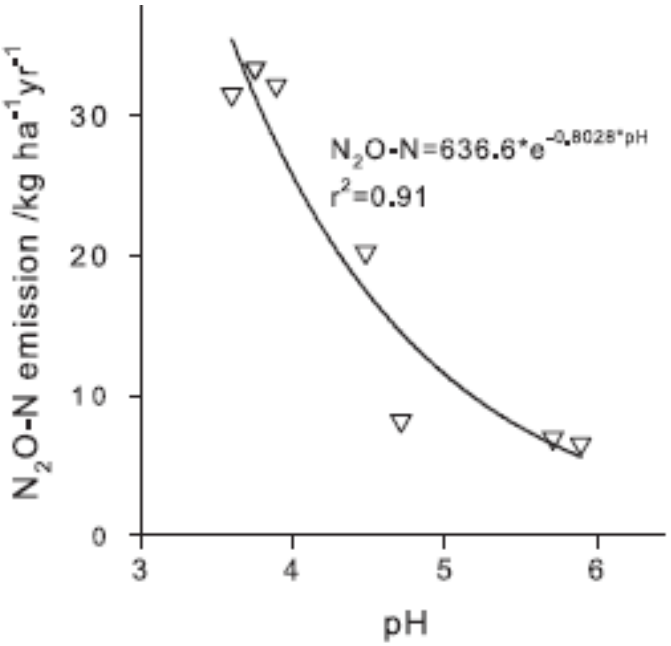

Figure 2.7 Nitrous oxide emissions plotted against soil pH adapted from (Weslien et al, 2009).

2.3.6 Organic matter content

Microbes rely on a carbon (C) supply as it provides them with the necessary substrate to grow (Rolston 1981). Microbial activity increases in the soil when there is readily available organic matter which can be metabolised (Rolston 1981; Fillery 1983; Sherlock et al. 1992). As denitrification and nitrification are respiratory processes, they require an organic substrate which can be oxidised. The rate and amount of denitrification will be influenced by the amount of C, its position in the soil profile, and its position in relation to NO_3^- or NO_2^- (Rolston 1981). In most soils, the organic matter content will be higher at the surface of the profile leading to decreasing denitrification rates with depth (Rolston 1981). Burford and Bremner (1975) found that N_2O production was positively correlated with total C, and highly correlated with water soluble organic C (Figure 2.8). Furthermore, water soluble C was

completely used by the denitrifying bacteria which caused the N_2O higher emissions. Myrold & Tiedje (1985) found in a loam soil when NO_3^- concentrations were low, low denitrification rates occurred due to a C supply limitation. Luo et al (2010b) found in two free draining soils, higher N_2O emissions from the free draining soil which had higher carbon content. This explains why peat soils have higher N_2O emissions (97-165kg N_2O-N/ha/year) compared to mineral soils (1-4kg N_2O-N/ha/year) (Sherlock et al. 1992).

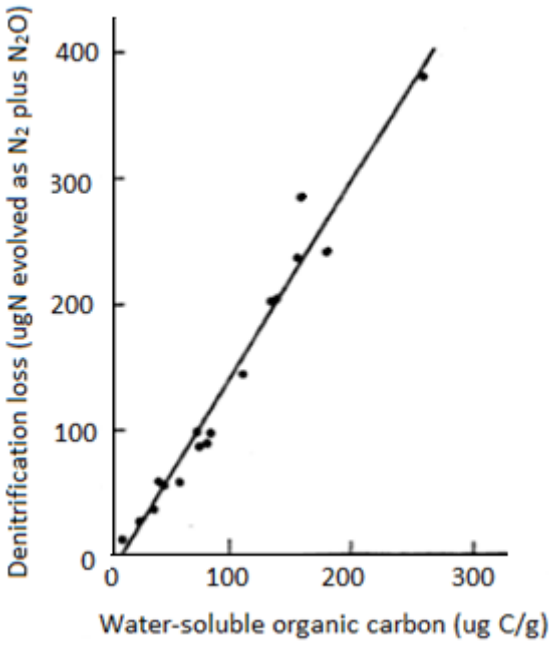

Figure 2.8 Relationship between denitrifcaiton loss and water soluble organic carbon in 17 soils (Burford and Bremner, 1975).

Plants can also influence N_2O emissions through their effect on the total and soluble organic C. As most N_2O emissions come from the surface of the soil, plants can greatly enhance N_2O emissions through the release of low molecular weight organic compounds into the rhizosphere (Thomson et al. 2012). This enhances heterotrophic activity and in addition, denitrifiers and nitrifiers are thought to also utilise this C. Enwall et al. (2007) found in their study of unfertilised bare soils and unfertilised cropped soils that plants enhanced nitrification. This is thought to be caused by higher organic matter which increases the N turnover in soils. However, this varies within plant species due to different N uptakes or root respiration (Philippot et al. 2009). Lower nitrifier activity can be caused by decrease in soil NH_4^+ concentration due to plant uptake or by higher competition by heterotrophic microbes in a C rich soil.

2.3.7 Ammonium and nitrate concentrations

Increasing NH_4^+ and NO_3^- concentrations increases the substrate available for microbial nitrification and denitrification. Avrahami et al. (2002) found that N_2O emissions increased with higher concentrations of NH_4^+, with nitrifiers contributing a higher proportion of N_2O emissions with increasing concentrations. They suggested that the enhanced nitrification rates provided NO_3^- which could then be further reduced by denitrifiers and both processes then contributed to higher overall N_2O emissions. However, Wetselaar et al. (1972) found that nitrification was inhibited in NH_4^+ concentrations above 3000ppm (Figure 2.9). Hynes and Germida (2012) found that AOB community composition changed with N bioavailability under harvested forest sites. Avrahami et al. (2002) found that bacteria adapted on a physiological level to increasing NH_4^+ concentrations, rather than shifting the community structure, hence narrowing the nitrification process to a few genetically diverse species. In contrast to bacteria, archaea have been found to be less active in high N environments, such as under urine patches (Di et al. 2010b).

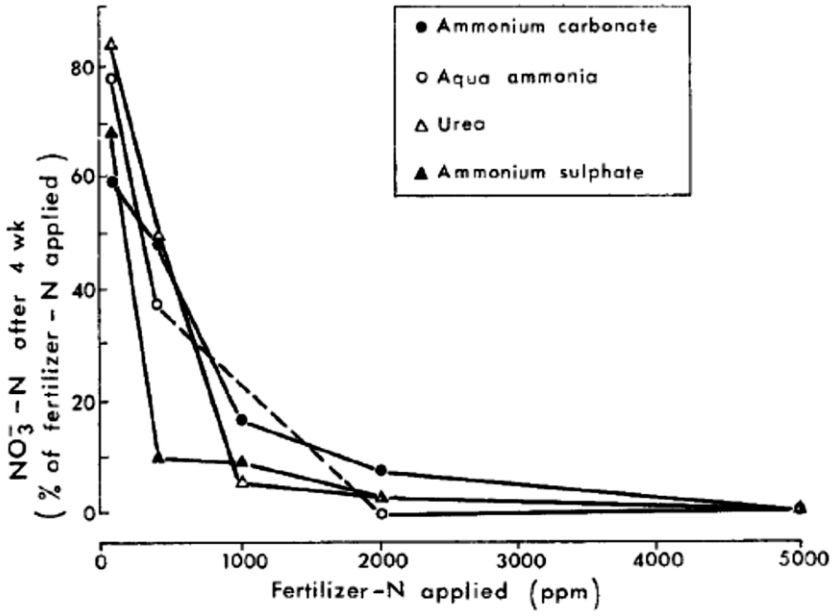

Figure 2.9 Percentage nitrate-N formed in four weeks as a function of various fertiliser-N applied to a clay soil (Wetselaar et al, 1972).

When soil organic C is not limiting, denitrification fluxes are proportional to soil NO_3^- concentrations (Sherlock et al. 1992). When the NO_3^- is highly available compared to the organic substrate, N_2O production is favoured (Delwiche 1981). Similar to pH, NO_3^- has more of an effect on the N_2O reduction to N_2 (Burford & Bremner 1978). In low NO_3^-

concentrations the reduction of N_2O to N_2 is delayed, whereas in very high concentrations the reduction is inhibited. This is caused by the inhibition of the N_2O-reductase enzyme (Fillery 1983) which causes incomplete denitrification and the production of N_2O.

2.4 Management effects on emissions

2.4.1 Fertiliser addition

Fertiliser N is produced synthetically through the Haber-Bosch process whereby atmospheric N_2 is converted to NH_3. Fertiliser addition to pasture increases N_2O emissions by providing a substrate for nitrification and denitrification to occur (Mosier et al. 1998). Cardenas et al. (2010) found that annual cumulative N_2O emissions were significantly related to quantity of fertiliser-N applied (Figure 2.10), with higher emissions when N-inputs exceeded plant demand. This is demonstrated by Rafique et al. (2011) who found that when fertiliser N was applied at a rate of 300 kg/N/ha, N_2O emissions were 5 kg N_2O-N/ha/year. However, N_2O emissions doubled when the rate increased to 400 kg/N/ha.

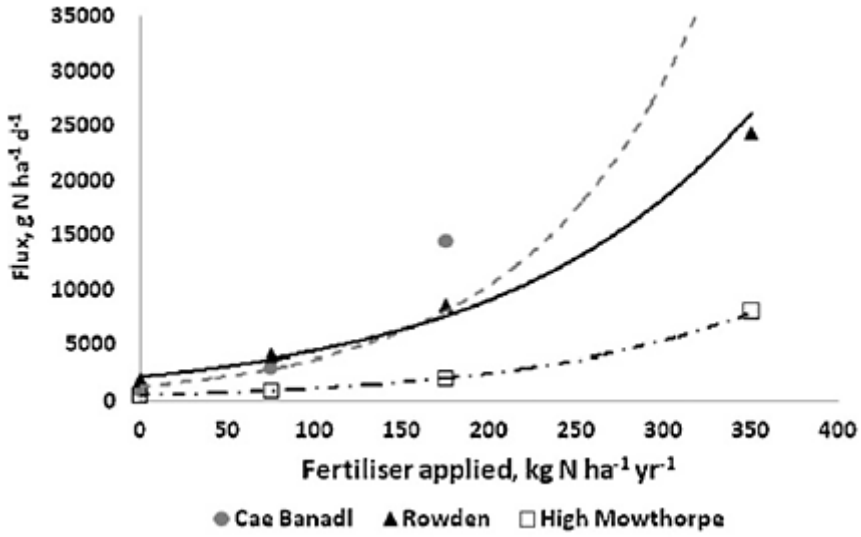

Figure 2.10 N_2O flux related to fertiliser-N applied to three study sites (Cardenas et al., 2010).

The use of fertilisers in agriculture can also increase the N_2O emissions in the long term, with around 0.2-4% of fertiliser applied lost as N_2O (Kroeze 1994). However, this figure is dependent on the soil type and properties, season and fertiliser used. Luo et al. (2007) found that urea fertiliser increased N_2O emissions for 30 days after its application. Cardenas et al. (2010) found varying N_2O emissions occurring at differing sites due to changes in soil type

and climate (Figure 2.10). The fertiliser type can also alter N_2O emissions with anhydrous NH_3 fertilisers resulting in higher emissions than NH_4^+ and urea fertilisers, and NO_3^- fertilisers showing the lowest emissions (Kroeze 1994). Eckard et al. (2006) found in their model that N fertiliser application is positively correlated with N_2O emissions, with higher emissions from NO_3^- and lower from urea and increasing separation between the two fertilisers modelled as the application rate increases (Figure 2.11). Eckard et al. (2006) explains this is occurring due to differences in the contribution of nitrification and denitrification to N_2O emissions.

When applying fertiliser to soils, climate and soil factors during application time can influence N_2O emissions by creating optimal soil conditions for N_2O production (Luo et al. 2010a). Limiting the amount of N fertiliser applied during late autumn/winter or early spring can decrease N_2O emissions from grazed pastures. This is because during these periods the soil is wet and pasture growth is slow, leading to low N consumption by plants and high denitrification rates. Furthermore, by avoiding application during these wetter, cooler months, there is also a reduction in the indirect losses of N_2O from the leaching of fertiliser N (Ledgard et al. 1988).

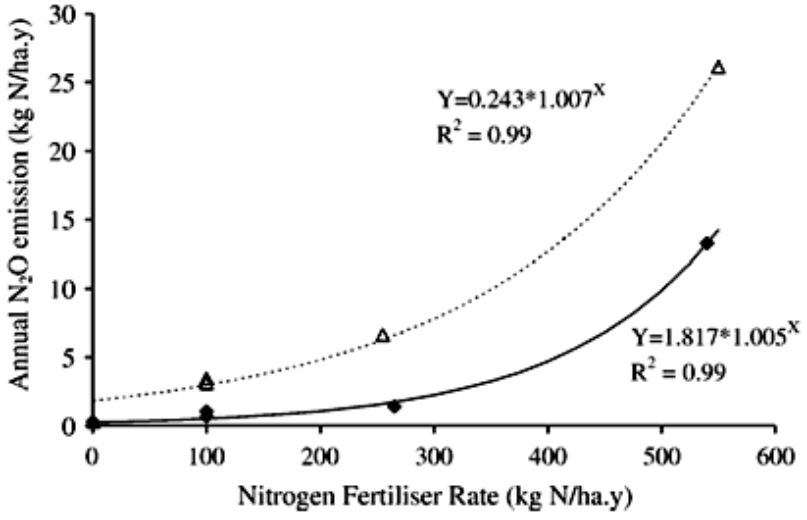

Figure 2.11 Predicted annual N_2O emissions with increasing N fertiliser application rates with two different fertilisers; urea (closed diamond) and nitrate (open triange) (Eckard et al., 2006).

Appling fertiliser at lower rates can decrease the N_2O emissions from soil, through reduced substrate availability. Lower application rates allows plants to only uptake what they need, enhancing plant growth and increasing N-use efficiency (Eckard et al. 2006). This further

lowers the substrate available for microbial nitrification and denitrification as the N is being removed from the soil. Eckard et al. (2006) found in there model that application rates exceeding 400kg N/ha/year provided diminishing returns for perennial ryegrass and clover.

2.4.2 Grazing

In New Zealand, the majority of agricultural N_2O emissions are caused by the deposition of animal excreta during grazing (de Klein & Ledgard 2005). Animal excreta deposition enhances N_2O production through the addition of N to the soil. Urine and dung patches create localised areas of high N concentrations, and in some urine patches the equivalent of 1000kg N/ha is deposited (Di & Cameron 2000). These high concentrations are excreted as the N ingested is more than what is required by the animal, and for dairy cows around 75% of N ingested is excreted (Reijnan 2002). These N 'hotspots' lead to N losses through leaching, denitrification, and NH_3 volatilisation as the concentration exceeds the utilization capacity of the plants and soil microbes (Eckard et al. 2010). The majority of N excreted is urea, which when deposited, forms NH_4^+ through hydrolysis, which is available for nitrification (Di et al. 2010a). The NO_3^- produced can then be subjected to various processes including plant uptake, leaching, and denitrification. These high levels of NH_4^+ and NO_3^- tend to accumulate in urine patches which create ideal conditions for N_2O production from denitrification and autotrophic nitrification (Sherlock et al. 1992). N_2O emissions from animal excreta in New Zealand predominantly occurs during autumn and winter from urine patches (de Klein et al. 2006). This is caused by a high rainfall leading to higher soil WFPS causing anaerobic conditions and consequently denitrification. Luo et al. (2008a) found that soils with urine addition produced higher N_2O fluxes for 6 weeks after application, with higher fluxes during the wetter months.

Rafique et al. (2011) found that N_2O emissions from frequently grazed sites were consistently higher than those emissions from less frequently grazed sites, with intensively grazed sites having three times the hourly N_2O emissions than extensively grazed sites. The authors state this is caused by the higher urine and dung deposition and soil compaction. Soil compaction by animal trampling is one of the major factors responsible for physical soil degradation in grazed pastures (Bhandral et al. 2007). Cattle hooves can exert static pressures of up to 400kpa and when the soil is wet this can cause severe damage to soils. Compaction reduces the physical integrity of the soil by modifying soil porosity, causing impediment of water, gas and nutrient movement (Bhandral et al. 2007). Furthermore it can reduce root elongation and

the mineralisation of carbon and nitrogen due to low oxygen concentrations. Compaction can enhance N_2O emissions directly by decreasing soil porosity and hence aeration and indirectly by carbon and nitrogen transformations (Bhandral et al. 2007). The decrease in soil porosity increases WFPS and decreases air filled pores (Smith et al. 1998), restricting oxygen diffusion and leading to anaerobic conditions and higher N_2O production (Bhandral et al. 2007). Eriksen et al. (2010) states that high N_2O emissions from grazed pastures are directly linked to soil compaction as well as fertiliser-N and excreta-N addition. Similarly, Oenema et al. (1997) assumes that compaction can cause a doubling of N_2O emissions under grazed pasture.

Urine deposition to pasture can alter other soil properties which in turn can alter soil microbial communities (Orwin et al. 2010). Urine deposition adds large quantities of N to the soil which can stimulate denitrifier and nitrifier activity. However urine can also provide other microbial resources such as labile carbon, and alter soil properties such as pH. The majority of N in urine is in the form of urea, and when hydrolysed by the enzyme urease, NH_4^+ and hydroxide ions (OH^-) are formed (Figure 2.12). As a result, urine patches become localised areas of high pH which favours the formation of NH_3 in the NH_4^+ to NH_3 equilibrium (Haynes & Williams 1993). Urine already has a high pH (pH 8.6) due to the high salt content. This high pH enhances urea hydrolysis when it enters the soil as the enzyme urease has a similar optimum pH. During the first 24 hours after urine deposition the pH increases rapidly at the soil surface and a rise of three units is not uncommon in the top 0.5cm (Figure 2.13). However as the nitrification process continues the pH will begin to decline. The initial high pH can enhance N losses through NH_3 volatilisation. NH_3 volatilisation is mainly affected by the supply of NH_3 through the NH_4^+ to NH_3 equilibrium and during high pH, high temperatures, and low water content, NH_3 production is favoured (Haynes & Williams 1993). NH_3 volatilisation can cause N losses of up to 22% in summer, 25% in autumn and 12% in winter (Sherlock & Goh 1984). This change in losses is caused by temperature and precipitation fluctuations. Losses through NH_3 volatilisation are important for N_2O emissions as they decrease the substrate available for nitrification and denitrification.

$$\mathbf{NH_3 + H_2O \leftrightarrow NH_4^+ + OH^-}$$

Figure 2.12 The ammonium to ammonia equilibrium (Haynes and Williams, 1993).

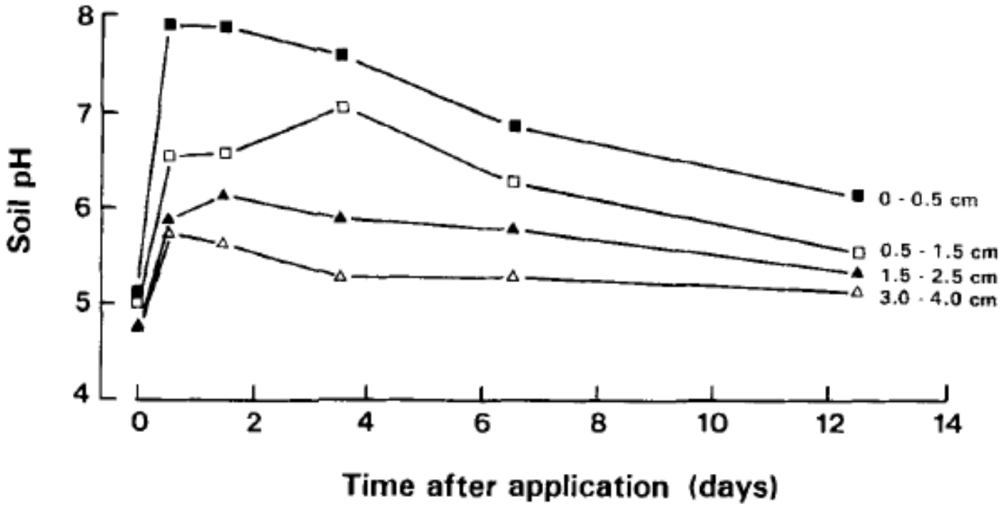

Figure 2.13: Mean daily pH at four depth increments under a urine patch (Haynes & Williams 1993).

2.5 Mitigation options

Mitigation of N_2O emissions is important for New Zealand's commitment to the Kyoto protocol and to reduce the impact of agriculture on the environment. The main sources of N_2O are biological fixation, animal excreta, and fertiliser through the microbial processes of nitrification and denitrification. Eckard et al. (2006) suggests that the key factors which affect N_2O emissions from grazing systems are soil aeration and NO_3^- levels. De Klein & Ledgard (2005) state that N_2O mitigation can be achieved either by reducing the addition of N to the system, by utilising it more efficiently, or avoiding soil conditions which favour those emissions. Thus, strategies to improve soil properties and the efficiency of N cycling in productive systems could lower N_2O emissions (Eckard et al. 2010).

N_2O emissions from denitrification and nitrification are closely linked to other N transformations. Hence a reduction in one process could potentially enhance other agricultural environmental issues (de Klein et al. 2001) e.g. NO_3^- leaching and NH_3 volatilisation. Hence, when mitigating N_2O emissions the whole agricultural nitrogen cycle must be considered.

2.5.1 On farm management

Animals provide a good method of harvesting N but their redistribution is inefficient (C. de Klein, personal communications, 01 April 2011). Urine patches provide localised

concentrations of N which are mainly lost in the system because the concentrations are too high for the plant to utilise (Eckard et al. 2010). Reduction in stocking rate is one method to lower N applications to the soil. By reducing the stocking rate, localised N inputs are reduced and consequently lower N_2O emissions. However, with continuing intensification and the increasing demand for animal protein (Luo et al. 2010a), lowering stocking rates will decrease farm productivity. Hence the use of diet manipulation, selective breeding, plant manipulation, and inhibitors is favoured to enhance system N efficiency without reducing stocking rates.

During the autumn winter period (April to August) around 80% of the annual N_2O emissions from grazed dairy pasture occurs (Ledgard et al. 1996). This is caused by higher rainfall and water filled pore space resulting in anaerobic conditions (de Klein et al. 2006). Reducing excreta N application during these wetter months can subsequently reduce the N_2O emissions significantly.

2.5.1.1 Diet Manipulation

Diet manipulation or additives can be used to reduce the amount of N in an animal's urine, thus reducing N addition to soil without reducing stocking rate (Eckard et al. 2010; Luo et al. 2010a). De Klein & Ledgard (2005) state that by supplementing grass-clover pasture with low N supplements such as maize or cereal silage, instead of boosting pasture growth with fertiliser during periods of pasture shortage, has the potential to decrease N_2O emissions by 27% per farm. Luo et al. (2008b) found that integrating a low protein feed such as maize silage, reduced N_2O emissions per kg of milk solids by 22% without reducing stocking rate. Using high sugar grasses has been proposed to reduce N concentrations in the urine and dung. High sugar grasses are hypothesised to decrease the C:N imbalance in the rumen, providing rumen-microbes with higher carbon, leading to N utilisation, rather than excretion (Ellis et al. 2011). Vanvuuren et al. (1993) found in their field study that dairy cows fed on a low protein/high sugar content perennial ryegrass diet had lower urine N concentrations than those fed on an all grass diet. Miller et al. (2001) found dairy cows who consumed high sugar cultivars reduced urine-N concentrations and increased milk production. They hypothesised that rather than the N being excreted, the N was used by the cow for the production of milk. Edwards et al. (2007) argues that although high sugar cultivars have been shown to decrease urine N, the possible changes in root exudation of sugars or the C:N ratio in the soil could alter microbial interactions. Hence further research is needed to determine whether these changes will mitigate or enhance GHG emissions.

Another method of diet manipulation is salt addition to feed (Luo et al. 2010a). This reduces the urine N concentration by causing the animal to drink more water. Animals then urinate more frequently with a more diluted urine N concentration, spreading the urine more evenly across the fields (Eckard et al. 2010). This decreases N losses through N_2O emission and NO_3^- leaching. However this area lacks field measurement of N_2O emissions influenced by salt supplementation and requires further research (Eckard et al. 2010).

Condensed tannin addition to feed results in a more efficient digestion of amino acids by forming complexes with proteins in the rumen (Eckard et al. 2010). Misselbrook et al. (2005) found that cows fed with high tannin feed had a lower urine N compared to those fed the low tannin feed, but they had higher N in the dung. With high tannin concentrations there is a shift in the proportion of N excreted, causing more to be deposited in the dung rather than the urine. Dung N is mainly in the organic form and is therefore less volatile and more slowly decomposed, especially when the tannin protein complex is present (Eckard et al. 2010). This leads to a decrease in N losses as urine N is reduced, lowering leaching and N_2O emissions.

2.5.2 Selective breeding

Urine patches create high localised concentrations of N in soil. This is due to the high N content of the urine but also due to the rapid application in one location. Producing animals which urinate more frequently (i.e. have a smaller bladder) or walk while urinating can spread the urine more evenly, reducing those high localised concentrations of N (Eckard et al. 2010). Genetic manipulation or improved animal breeding could improve N conversion in the rumen, reducing concentrations in the urine (Eckard et al. 2010). Similarly, breeding animals which use their N intake more efficiently, for example, for milk production, will reduce the amount of N excreted, thereby reducing N losses and hence N_2O emissions. Bell et al. (2011) states that future policy should aim to select dairy genotypes with improved feed utilization efficiency for milk production rather than low forage diets. Selective breeding offers a medium to long term solution to greenhouse gas mitigation (Wall et al. 2008); however its success depends on the producers being committed to its implementation.

2.5.3 Plant manipulation

Like animals, plant characteristics can be manipulated to decrease N_2O emissions. By increasing rooting depths, tannin content, water soluble carbon content and residue quality, N_2O emissions can be reduced (Luo et al. 2010a). Crush et al. (2007) found in ryegrass

pasture a strong positive correlation between rooting depth and NO_3^- interception. Longer roots increase the plants ability to remove NO_3^- from greater depths, reducing N losses. By removing the NO_3^- in the soil, N_2O emissions are reduced by decreasing the substrate available for denitrification. Similar to tannin addition to feed, plants with higher tannin content can cause less N to be excreted in the urine relative to the dung (Misselbrook et al. 2005).

2.5.4 Nitrogen process inhibitors

2.5.4.1 Urease inhibitors

Urease and nitrification inhibitors can be used as effective mitigation options to control N losses from urine and N fertiliser (Luo et al. 2010a). High losses of N can occur directly after fertiliser application if it is not incorporated into the soil almost immediately (Chen et al. 2008). Nitrogen is lost by NH_3 volatilisation which occurs when urea is converted to NH_3 at the soil surface through the enzyme urease. To prevent high losses, one approach to decrease ammonia volatilisation is to use a urease inhibitor (Chen et al. 2008). These work by slowing the urea hydrolysis (Luo et al. 2010a) allowing the urea to move into the soil, and be converted to NH_4^+, which can be retained by the soil colloids. Urease inhibitors have been proven to delay urea hydrolysis and increase productivity under pasture and cropping systems (Chen et al. 2008). Preventing or slowing urea hydrolysis can lead to decreased N_2O emissions through the reduction in NH_4^+ supply. Dawar et al. (2011) found a reduction in N_2O emissions of 7% when using the commercial product Agrotain which contains the urease inhibitor thiophosphoric triamide. Zamen et al. (2009) found that the same urease inhibitor reduced ammonia volatilisation but did not reduce N_2O emissions. However when applied in conjunction with the nitrification inhibitor, DCD, it reduced N_2O emissions by 37%, 67% and 28% in autumn, summer and spring respectively. This was higher than when DCD was applied alone. This was caused by the urease inhibitor limiting the availability of the ammonium combined with the NH_4^+ retention by the DCD (Zaman et al. 2009).

2.5.4.2 Nitrification inhibitors

Maintaining N in the NH_4^+ form prevents N losses through nitrification and denitrification (Chen et al. 2008). This can be done with the use of nitrification inhibitors which slow down the enzymatic conversion of NH_4^+ to NO_3^-, reducing NO_3^- leaching and the formation of N_2O. The most common nitrification inhibitors are nitrapyrin and dicyandiamide (DCD) (Di & Cameron 2002). DCD is used more frequently as it is cheap to produce and has high water

solubility which allows it to be applied in a liquid form or fine particle suspension. It is also less volatile than nitrapyrin, which means it can be used in conjunction with solid fertilisers and decomposes in the soil to form NH_4^+ and CO_2 (Amberger 1989).

By preventing the conversion of NH_4^+ to NO_3^-, nitrification inhibitors increase the potential of the N applied to the soil to be taken up by plants. This decreases N losses through leaching as NH_4^+ is a cation which means it can be adsorbed on to the negatively charged soil colloids, immobilised by organic matter or fixed onto clay minerals (Di & Cameron 2002). Nitrification inhibitors affect both the nitrification and denitrification production of N_2O as they inhibit nitrification and reduce the substrate available for denitrification. Nitrification inhibitors can have a significant impact on N_2O emissions from urine patches, and when applied across grazed pasture can greatly decrease the N_2O emissions from agricultural land (Di & Cameron 2002). Di & Cameron (2002) found in their lysimeter study that the application of DCD reduced N_2O emissions by 82% and autumn NO_3^- leaching by 76% during their eight month trial. Similarly, Di & Cameron (2006) found a reduction of N_2O emissions by 56-73% when DCD was applied as a fine particle suspension on two different soils. Smith et al. (2008) found granular DCD to be effective at reducing N_2O emissions by 57-97% from grazed pasture in Southland. DCD also results in higher herbage yield as the N remains as NH_4^+ in the soil for longer, allowing more plant uptake and lower N losses (Di & Cameron 2006).

2.5.4.3 Factors affecting nitrification inhibitor effectiveness

2.5.4.3.1 Rainfall

The major challenge for nitrification inhibitor application is ensuring that it enters the whole top soil layer which is microbiologically active (Di & Cameron, 2006). This guarantees that urine will not pass the nitrification inhibitor after deposition. Using a fine particle suspension or solution of DCD has overcome this (Di & Cameron, 2006). However in soils under high rainfalls the loss of DCD can reduce its effectiveness, as the DCD is leached down the profile resulting in separation from the NH_4^+ applied at the soil surface (Abdelsabour et al. 1990). This is because DCD is a non-ionic compound hence will move with water flow; however NH_4^+ is retained due to its ionic properties. When DCD is leached down the profile into low microbial active subsoils it can prolong for longer. Shepherd et al. (2012) found DCD being leached 15 months after application. They assumed this was due to its movement down the profile where microbial degradation was low. Furthermore, they found that leached DCD was directly related to cumulative drainage, with DCD leaching losses tripling under high rainfall

(2280mm) in a silt loam. Luo et al. (2010b) state that under heavy winter rainfall the effectiveness of DCD may be reduced. This is due to its movement out of the microbial 'active' area of the soil or being leached completely. Furthermore, leaching of DCD may have further implications as DCD could enter waterways, interrupting water N-cycling processes.

2.5.4.3.2 Soil temperature

DCD is used as a nitrification inhibitor because it can be broken down by microbes into C and N and thus does not prolong in the environment. However, in soils where microbial activity is high, DCD can be degraded too quickly for it to work effectively. In the tropics, high soil temperatures increase microbial activity which reduces DCD's half-life, meaning that DCD has to be used in high concentrations (Puttanna et al. 1999a). Amberger (1989) found that at 12˚C DCD decomposed completely by 12 weeks, in contrast at 4˚C concentrations were still relatively high at 17 weeks. Puttanna et al. (1999a) found that DCD had less inhibition effect at 30˚C compared to other nitrification inhibitors. Di and Cameron (2004) found in their incubation trial that the half-life of DCD increased 5-fold when the temperature increased from 8^0C to 20^0C. From this, they concluded that applying DCD during later autumn-winter-early spring period in New Zealand when the soil temperature is less than 10^0C would extend the time period DCD remained effective in the soil.

2.5.4.3.3 Organic matter

When evaluating the effectiveness of DCD in the soil, the soil organic matter content must be considered (Reddy & Datta 1965). Soils with higher organic matter have been shown to increase the degradation of DCD. Reddy (1964) found a more rapid decomposition of DCD in a fine sandy loam than a coarse sandy loam, due to the higher soil organic matter content. Similarly, Singh et al. (2008) found that the maximum inhibition effect was in soil with the lowest organic C content, with the half-life of DCD decreasing when the organic C doubled. The reduction in DCD's half-life with higher organic C content can be attributed to higher microbial activity, which decomposes the DCD (a readily accessible C-source) to NH_4^+ faster (Reddy 1964).

2.5.4.3.4 Soil pH

Increasing the pH of the soil reduces DCD's efficiency (Puttanna et al. 1999a). This is thought to be due to an increase in general microbial activity at a high pH which enhances DCD's break down. Mahmood et al. (2011) found that DCD enhanced N losses from urea in

an alkaline calcareous soil. They suggest this is due to increased NH_3 volatilisation from the accumulation of NH_4^+ in the soil. Research in DCD's effectiveness in varying soil pH's, especially under New Zealand's climate is limited, and further studies need to be undertaken.

2.6 Conclusions

Nitrous oxide emissions are produced through the process of nitrification and denitrification. Ammonia oxidation produces the substrate available for denitrification, thus is the rate determining step for N_2O emissions. Ammonia oxidation is carried out by bacteria and archaea, however bacteria dominate the process under most soil conditions (Di et al. 2009). Archaea are thought to be more dominant in low nutrient, low pH environments (Leininger et al. 2006; Di et al. 2010b). However, studies which support this hypothesis (Nicol et al. 2008; Shen et al. 2008; Zhang et al. 2011), are carried out under natural or long term pH variations. Few studies determine the change of AOB and AOA communities under short term pH changes and less establish the effect of community changes on N_2O emissions.

Various soil conditions can alter N_2O emissions through enhanced substrate production and/or the inhibition of N_2O-reductase. Soil pH has been shown to alter N_2O emissions with greater emissions from acidic soils (Clough et al. 2004; Weslien et al. 2009). It is hypothesised that acidic pH causes inhibition of N_2O-reductase (Fillery 1983) as well as greater substrate availability as NH_4^+ is favoured over NH_3 (de Boer & Kowalchuk 2001). However, denitrification rates and ammonia oxidation have been shown to be reduced in acid conditions. This is caused by the high pH optima (pH 7-8) of nitrifiers (Rolston 1981; Sherlock et al. 1992) and the unavailability of NH_3 (de Boer & Kowalchuk 2001). Studies which have found differences in N_2O emissions with soil pH have been in natural or long term soil pH's or manipulated in incubation studies; few studies have identified changes in N_2O emissions (and N-cycling) and ammonia oxidising communities together, and in a field trial.

Soil aggregate size has been hypothesised to affect N_2O emissions by altering the aeration of the soil. Large aggregates are thought to have an anaerobic centre caused by low oxygen diffusivity. However, studies argue what compaction and aggregate size is required for an anaerobic centre or environment to be created (Sexstone et al. 1985; Uchida et al. 2008; Diba et al. 2011). N_2O emissions are enhanced under anaerobic conditions, through both denitrification and nitrification processes. Thus larger aggregates, with larger anaerobic zones should have higher N_2O emissions. This was supported by Diba et al. (2011) but conflicted with Uchida et al. (2008), who state that smaller aggregates produce higher N_2O as they can be compacted tighter. In these studies only N_2O emissions were measured and the effects on microbial communities were not determined. Since the production of N_2O is predominantly a

microbial process, logic suggests that microbial communities should be accessed when trying to explain differences in N_2O emissions.

Various management practices can lead to enhanced N_2O emissions (de Klein & Ledgard 2005). Agriculture is accountable for the majority of N_2O emitted and the increase in N_2O emissions in recent years. In New Zealand, year round pastoral farming means that excreta deposition is the main cause of enhanced emissions (de Klein et al. 2006). Localised areas of high N loading, caused by urine and dung patches, create ideal conditions for N_2O production. The nitrification inhibitor, DCD, has been promoted as a method to mitigate N_2O emissions from agricultural soils and studies have demonstrated its effectiveness (Di & Cameron 2005, 2006; Singh et al. 2008; Smith et al. 2008; Luo et al. 2010b; de Klein et al. 2011; Di & Cameron 2011). Rainfall, temperature, organic carbon content and pH have been identified as soil properties which affect DCD's effectiveness. Few studies have detailed the effect of pH on DCD's inhibition abilities with Puttanna et al. (1999b) stating that increasing the pH reduces DCD efficiency, and Mahmood et al. (2011) state the application of DCD enhanced N losses in a high pH soil. Field studies identifying soil properties effects on DCD are limited, especially in New Zealand, and none have determined the effect of a short term pH change, or aggregate size variation on DCD's effectiveness.

Gaps in our knowledge of aggregate size and pH change on N_2O emissions, ammonia oxidising communities, and DCD effectiveness, have been identified in this literature review. Therefore the objectives of this thesis are to:

1. Quantify nitrous oxide emissions from an agricultural soil with different soil aggregate size and soil pH;

2. Determine the effectiveness of the nitrification inhibitor DCD in reducing nitrous oxide emissions from soil over a range of aggregate sizes and soil pH values;

3. Determine changes in AOA and AOB abundance in agricultural soils with changes in soil aggregate size and soil pH.

Chapter 3
Materials and Methods

3.1 Incubation trial

3.1.1 Experimental design

To test the proposed hypothesis, that aggregate size will effect N_2O emissions, ammonia oxidising communities, and DCD effectiveness, an incubation trial was implemented.

An incubation study investigated the effect of aggregate size on N_2O emissions, ammonia oxidising communities, and DCD effectiveness. A randomised block design was chosen to reduce the effect of airflow and temperature variation within the incubator. A Temuka clay loam soil was collected from Lincoln University. Prior to starting, the field capacities of the sieved aggregates were calculated. This allowed the soil water content to be maintained, with the addition of deionised water, at field capacity throughout the trial. Twelve treatments were blocked in four replicates (Figure 3.1). For N_2O emissions, glass jars with a 575mL volume were used. To eliminate disturbance of the soil in the incubation jar, separate soil sampling tubes were used for mineral nitrogen (N), ammonia oxidising bacteria (AOB) and ammonia oxidising archaea (AOA) assays, and soil moisture. There was an accompanying soil sampling tube for each sampling date (12 batches), and for each incubation jar (Figure 3.2). Centrifuge tubes with a 50mL volume were chosen as they allowed enough soil for one sampling date to be packed to the correct bulk density ($1.0 g/cm^3$).

3.1.2 Trial set up

The incubation experiment was set up in February 2012, with Temuka clay loam soil randomly collected in two batches (November 2011 and February 2012) at a depth of 0-10 cm from the field study site at Lincoln University, Christchurch (43˚38’55”S, 172˚ 28’4”E). For detailed soil characteristics see Appendix 1. The two batches were mixed well and stored in the laboratory at 4^0C until sieved. Soil aggregate were sieved into three fractions; large (4-5.6 mm), medium (2-4 mm) and small (1-2 mm). Sieves were stacked on top of each other to allow the fractions to separate while sieving. Fractions were then well mixed and stored at 4^0C until trial set up.

Soil fractions were packed to a bulk density of 1.0 g/cm^3 in both the incubation jars and the soil sampling tubes. To allow airflow within the jars and tubes, incubation jar lids with two holes and parafilm with a hole punctured in the top was used for the jars and tubes respectively (Figure 3.2). Soil sampling tubes and incubation jars were then placed in an incubator set to 10^{0}C for one week to allow the microbial community to equilibrate before treatments were applied. The incubation temperature of 10^{0}C was selected as it represents a typical New Zealand winter temperature, when the soils are wet and N_2O emissions are at their highest (Di et al. 2010a).

1 Large Control	**5** Small DCD	**9** Medium DCD	**13** Large Urine	**17** Large Control	**21** Large DCD	**25** Small DCD	**29** Large Control	**33** Medium Urine	**37** Small DCD+Urine	**41** Large Urine	**45** Small DCD
2 Small Urine	**6** Small DCD+Urine	**10** Small Control	**14** Medium Control	**18** Medium Urine	**22** Small DCD	**26** Large DCD	**30** Medium DCD+Urine	**34** Medium Control	**38** Large DCD+Urine	**42** Small Control	**46** Small Urine
3 Medium Urine	**7** Large DCD	**11** Large DCD+Urine	**15** Small Urine	**19** Medium DCD+Urine	**23** Large DCD+Urine	**27** Small Control	**31** Small DCD+Urine	**35** Small Urine	**39** Large Control	**43** Medium Urine	**47** Medium DCD+Urine
4 Medium Control	**8** Medium DCD+Urine	**12** Large Urine	**16** Small DCD+Urine	**20** Small Control	**24** Medium DCD	**28** Medium DCD	**32** Large DCD+Urine	**36** Large Urine	**40** Medium DCD	**44** Medium Control	**48** Large DCD

----------FRONT OF INCUBATOR----------

Figure 3.1 Randomised block design used in the incubation study. Treatments were randomly allocated using a random number generator

Treatments were applied on 20th February 2012 using urine collected from dairy cows grazing at the Lincoln University Research Dairy Farm (LURDF). The following treatments were used;

- control,
- DCD (10 kg DCD/ha),
- Urine (700 kg Urine-N/ha), and
- Urine plus DCD (700 kg urine-N/ha + 10 kg DCD/ha).

Treatments are referred to as "control", "urine-only", "DCD-only" and "urine+DCD" respectively. For ease of treatment application, in the urine+DCD treatment, the DCD was dissolved in the urine before application. All treatments were applied to the surface of the incubation jars and soil sampling tubes (through the hole in the parafilm).

Figure 3.2 Top left: Incubation jars, for N_2O sampling, with incubation lids; bottom left: soil sampling tubes arranged by batch number in incubator; and right: Incubation soil sampling tubes with parafilm.

3.1.3 Field capacity and soil moisture

The field capacity of each soil aggregate size was measured prior to treatment application. A soil core with a volume of 209.3 cm^3 was packed to a bulk density of 1.0 g/cm^3 for each aggregate size treatment, and wet up until the soil was saturated. The core was then placed on a tension table at 1m suction (-1 bar) for 2 days. The soil was then weighed and dried and weighed again to calculate the field capacity using the following formula:

FC = (mass of water (g)/mass of dry soil (g))*100

Samples were maintained at field capacity throughout the trial by the addition of deionised water to maintain the required weight (601 g for jars and 61 g for tubes). Incubation jars were maintained twice a week with the addition of water, if required, after gas sampling. Soil sampling tubes were checked once a week and water added when needed.

Soil moisture was monitored during the trial by taking a subsample during soil sampling. Approximately 10 g of soil was taken and dried at 105^0C for 24 hours and reweighed (Blackmore et al. 1987). Soil moisture was calculated using the following formula:

Soil moisture (%) = ((wet soil (g) - dry soil (g))/dry soil (g)) x 100))

3.1.4 Nitrous oxide measurement

A modified method from Hutchinson and Mosier (1981) was used to measure N_2O emissions in the laboratory. Incubation jars were taken from the incubator in two batches for ease of sampling. Jars were lined across the bench with incubation lids removed (Figure 3.3) and gas sampling lids were lined up behind incubation jars. Gas sampling lids contained a septum; tap and needle (see Figure 3.3). The gas sampling lids sealed the incubation jar and allowed gas to build up in the head space prior to collection by a syringe. At time zero, gas sampling lids were placed on the incubation jar and an initial sample taken. Evacuated vials were placed on the gas lid and the extracted gas was pumped to a volume of 20 mL three times to allow gas mixing in the headspace. 15 mL of gas was then extracted and injected into the 12 mL glass vial. A second sample was taken after 30 mins. Nitrous oxide gas sampling started on day 1 (21 February 2012) and occurred twice weekly for the first 90 days, and then once per week for the remainder of the study. Prior to gas sampling, the vial septum was replaced and the vial evacuated of air. This was to ensure all non-sample N_2O was removed.

Figure 3.3 An example of gas sampling the incubation trial. Top left: the gas sampling lids used. Bottom left: A syringe was used to mix the gas and then inject 15mL into evacuated vial. Right: Gas sampling across the bench.

Nitrous oxide concentration was measured using a gas chromatograph (SRO8610 linked to a Filson 222XL autosampler) using an Electron Capture Detector (ECD) (SRI Instruments, USA) and quantified using stored ambient air samples. Elevated concentrations were achieved using ethylene and acetylene in stored air samples.

3.1.5 Extractable ammonium and nitrate

For each sampling date the required rack of tubes was removed from the incubator, the soil emptied into individual plastic bags and mixed well. A 5 g subsample was taken for extractable ammonium (NH_4^+) and nitrate (NO_3^-). Samples were stored at 4^0C until extracted to minimise the loss of NH_4^+. Ammonium and NO_3^- were extracted from the subsample by the addition of 25 mL of 2 M potassium chloride (KCl) (Blackmore et al. 1987). Samples were then placed on a shaker for one hour then centrifuged at 2000 x g for 10 mins. Samples were filtered using Whatman No. 41 filter paper and the collected filtrate frozen at -20^0C until analysed. Soil samples were taken once per week for the first month, then fortnightly thereafter. Ammonium and NO_3^- were analysed using a flow injector analyser (FIA) (FOSS FIAstar 5000 triple channel analyser) with SoFIA software version 1.30 (Foss Tecator AB, Sweden).

3.1.6 DCD

In total, only 24 samples contained and thus were analysed for DCD. From the sample, 5 g of soil was weighed into a centrifuge tube and 25 mL of deionised water added. Samples were placed on a shaker for 1 hour then centrifuged at 2000 x g for 20 min. The samples were then filtered using Whatman No. 41 filter paper and the collected filtrate frozen at -20^0C until analysed. Soil samples were taken once per week for the first month, then fortnightly thereafter. DCD concentration was analysed on a Shimadzu series High Performance Liquid Chromatography (Tokyo, Japan) using a cation-H guard column (Phenomenex, USA) and a 0.025M sulphuric acid mobile phase at a flow rate of 0.6 ml/min by UV detection at a wavelength of 210 nm.

3.1.7 AOA/AOB assays

3.1.7.1 DNA extraction

A subsample of soil was collected and stored at -80^{0}C for molecular analysis at all soil sampling dates. DNA was extracted from the soil using the NucleoSpin® Soil Kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions. A 0.25 g subsample of soil was weighed into a NucleoSpin® bead tube and 700 µL of solution SL2 and 150 µL of Enhancer SX was added to adjust conditions for cell lysis. Samples were then vortexed using MP Fast prep-24 (MP Biomedicals, USA) at 4.0 m/s speed for 1 min to homogenise the sample and then centrifuged at 11000 x g for 2 min. 150 µL of buffer SL3 was added and vortexed to mix before being incubated for 5 min at 4^{0}C. Samples were then centrifuged for 1 min at 11000 x g. Supernatant was collected and transferred to a NucleoSpin® Inhibitor Removal Column in a Collection Tube and centrifuged for 1 minute at 11,000 x g. The column was then discarded and to adjust binding conditions, 250 µL of Buffer SB added to the flow through and vortexed to mix. A NucleoSpin Soil Column was then placed in a new collection tube and 550 µL of sample was loaded onto the column. This was centrifuged for 1 minute and the flowthrough discarded. This was repeated until there was no remaining sample. To wash the silica membrane, 550 µL of Buffer SB was then loaded to the column and centrifuged for 30 seconds and the flowthrough discarded. This was repeated with 550 µL of Buffer SW1. Then 700 µL of Buffer SW2 was loaded to the column, vortexed for 2 seconds and then centrifuged for 30 seconds and the flowthrough discarded. This was then repeated. Once the flow through was discarded, the column and collection tube was centrifuged for 2 minutes to dry the column. The column was then transferred to a new collection tube and the DNA eluted using 100 µL of Buffer SE. DNA was stored at -20˚C until analysis.

3.1.7.2 PCR analysis

AOA and AOB ammonia monoxygenase gene (*amoA*) abundance was measured using real-time quantitative PCR (qPCR) on a Rotor-Gene^{TM} 6000 (Corbett Life Science). All PCR reactions were set up using a CAS-1200 Robotic liquid handling system (Corbett Life Science, Australia). All soil genomic DNA samples were diluted ten times with deionised water prior to use as a template in PCR reactions, as described by Di et al. (2010b). AOA and AOB amoA genes were quantified using the PCR primers amoA1F/amoA2R (Rotthauwe et al. 1997) and Arch-amoAF/arch-amoAR (Francis et al. 2005) respectively. A reaction

mixture of 20 µL was prepared using the CAS1200 and contained 10 µL of SYBR Premix Ex Taq (TaKaRa, Nori Biotech, Auckland, New Zealand), 0.4 µL of each primer (final concentration 0.2 uM) and 1.5 µL of 1:10 diluted template soil genomic DNA. The PCR was run according to the temperature profiles shown in Table 3.1. To confirm the PCR product specificity after amplification, a melting curve analysis was performed measuring the fluorescence continuously as the temperature increased from 50°C to 99°C. Data was then analysed using the Rotor Gene 6000 series software 1.7.

Standard curves for real-time qPCR were developed using the following process. Bacterial and archaeal amoA genes were amplified from the extracted DNA using the aforementioned primers. A PCR clean up kit (Axygen) was then used to purify the PCR products which were then cloned into the pGEM-T Easy Vector (Promega, Madison, WI). Following the manufacturer's instructions, the resulting clones were transformed in *Escherichia coli* JM109 competent cells (Promega). The transformed *E. coli* cells were grown on solid LB plates at 37°C overnight. Ten to fifteen bacterial colonies from the plate were then individually inoculated into a 3 mL LB broth medium and incubated overnight in an orbital incubator-shaker at 37°C and 250 rpm. The plasmids carrying correct amoA gene inserts were then extracted from bacterial cultures using QIA Prep Spin Miniprep Kit (Qiagen, Crawley, UK) and sent for sequencing. The plasmid DNA concentration was determined on a Qubit™ fluorometer (Invitrogen, NZ). The copy numbers of target genes were then calculated directly from the concentration of extracted plasmid DNA. To generate an external standard curve, tenfold serial dilutions of a known copy number of the plasmid DNA were then subjected to a real-time PCR assay in triplicate.

Table 3.1 PCR temperature profiles used for AOA and AOB, repeated for 40 cycles (Di et al. 2009).

	AOA		AOB	
First denaturing	94°C	2 minutes	94 °C	2 minutes
Denature	94 °C	20 seconds	94 °C	20 seconds
Anneal	55 °C	30 seconds	57 °C	30 seconds
Extend	72 °C	30 seconds	72 °C	30 seconds

3.2 Field study

3.2.1 Experimental design

To determine the effect of soil pH on N_2O emissions, ammonia oxidising communities, and DCD effectiveness, a field trial was employed. A randomised block design was used to reduce environmental and soil variability across the trial plot. Treatments were blocked in four replicates (Figure 3.7). Soil pH alterations began, with the addition of CaO and HCl, prior to urine and DCD treatment application to ensure the required pH was reached and maintained. A higher concentration of HCl and NaOH, instead of CaO, was used for additional applications after high precipitation. Soil sampling plots were used for mineral N, AOB and AOA assays, soil pH and soil moisture, and gas sampling plots were used for N_2O emissions. Soil sampling plots were kept separate to gas sampling plots to eliminate soil disturbance effects. Grass was cut prior to any treatment application and was cut throughout the trial when required.

3.2.2 Site selection

The field study was set up south of the lysimeter paddock at Lincoln University, Christchurch (43˚38’55”S, 172˚ 28’4”E) (Figure 3.4). The site has been under pasture for 8 years with on-going fertiliser and irrigation additions. Sheep grazing was the predominant land use. The soil was a Temuka clay loam and was the same soil (and collection site) used in the incubation study. For detailed soil characteristics see Appendix 1. A basal application of urea-N (50 kg/ha) and super phosphate fertiliser (500 kg/ha) was applied in December 2011 prior to the start of the study to maintain the microbial community and pasture growth.

3.2.3 Field study set up

The experimental site encompasses an area of approximately 115 m^2 with 48 treatment plots (4 replicates of 12 treatment plots) 1.5 x 1.3 m in size (Figure 3.7). The treatment plots contained a gas sampling ring with companion soil sampling plot and a buffer region of non-sampling area (25 cm) between each plot. The non-sampling area allowed room for sampling, and avoided cross contamination with pH treatments. A randomised block design was used in the study (Figure 3.7).

Figure 3.4 Study location in the paddock south of the lysimeter paddock, Lincoln University. Red area indicates approximate field study location.

Figure 3.5 Left: Field trial set up for application of pH treatments, pH treatments were applied over each of the 48 plots. Right: Final field trial set up, urine/DCD treatments were applied only within the gas and soil sampling rings.

3.2.4 pH treatment set up

The field trial was established on the 3rd of May 2012 according to Figure 3.7. The paddock was mown and the grass clippings removed prior to treatment application. The pH treatments were applied in three batches on the 4th, 7th and 9th of May 2012, using the following methods. For the basic treatment, 29.25 g of CaO was applied as a fine powder to the surface of the plot, and watered in with three 10 L aliquots of water using a watering can (Figure 3.6). This allowed the water to 'soak in' and prevented overland flow into neighbouring plots. For the acid pH treatment, 360 mL of 1M HCl was made up to 10 L with water and applied three times to each plot. The control pH plots had a total of 30 L of water applied. This method was repeated for each application date. The treatments were applied across the whole plot (1.5 x 1.3m), including non-sampling/buffer areas.

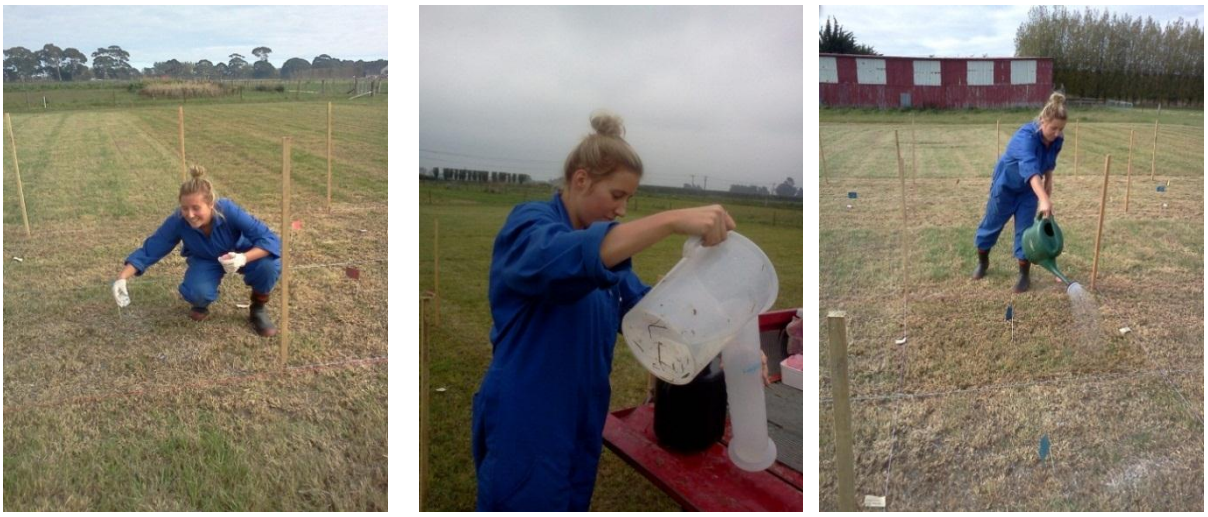

Figure 3.6 Application of pH treatments (left to right); applying the CaO, measuring out the HCl and applying the water to the plots.

After monitoring the pH for one month, the pH values reached satisfactory values of less than 5 for the acidic treatment and greater than 7 for the alkaline treatment. However, heavy rain and snowfall in early June 2012 leached out the acid/base treatments. To counteract this, on the 21th June 2012, 360mL of 10M HCl and 360mL of 1.2 M NaOH, rather than CaO, was applied to the acid and alkaline treatment plots respectively, using the same method above. Another application of NaOH was made on the 28th June 2012 to overcome the buffering capacity of the soil and reach the required alkaline pH. Unsatisfactory pH changes were experienced with the use of CaO due to its low solubility, hence NaOH was used instead. The pH treatments, HCl, water, and CaO/NaOH are referred to as acidic, native and basic henceforth.

Soil samples from each plot were collected weekly from the 11th May to the 30th July until the pH stabilised at <pH 5 for the acid treatment and >6.5 for the alkaline treatment. Three soil cores were taken from the perimeter of each plot to prevent damage to the middle where future gas and soil sampling would occur. A sub sample was taken for AOA and AOB abundance and stored in the -80^{0}C freezer until required for analysis. Further subsamples were taken for pH measurement and KCl extractions for NH_4^+ and NO_3^-. The remaining soil was stored at -20^{0}C

Figure 3.7 Field trial study design. Plots are 1.5 m x 1.3 m, pH treatment was applied across whole plot including 25 cm buffer non-sampling area (white).

3.2.5 Soil pH

The soil pH was measured by weighing a 10 g subsample of field moist soil into a 70mL vial and adding 25 mL of deionised water (Blackmore et al. 1987). This was shaken well and then left over night to settle before the pH was read using a Mettler Toledo Seven Easy pH Meter (Mettler Toledo, Switzerland). The soil pH was read for each plot weekly before the addition of urine and DCD treatments and then at soil sampling days after the treatment application.

The soil pH was monitored and reapplication of HCl and NaOH to appropriate plots occurred on the 18th September 2012. This was applied using the same concentrations and methods as described in section 3.2.4. However treatment application did not occur across the whole plot, but instead only in the soil and gas sampling rings.

3.2.6 Treatment application

Prior to treatment application, soil and gas sampling rings were installed in the plots (Figure 3.8). Treatments were applied on the 9th August 2012. The following treatments were used;

- Control,
- DCD (10 kg DCD/ha),
- Urine (700 kg Urine-N/ha) and,
- Urine plus DCD (700 kg urine-N/ha + 10 kg DCD/ha).

These are referred to as "control", "urine-only", "DCD-only" and "urine+DCD". Synthetic urine was used in due to the volume required. This was made using a mixture of urea (14 g/L), glycine (3.5 g/L), potassium bicarbonate (16 g/L), potassium chloride (3 g/L) and potassium sulphate (2 g/L) adapted from Fraser et al. (1994). Two litres of 'urine' was applied evenly across the urine and urine plus DCD treatment rings using a jug (Figure 3.9) and 2 L of water was applied to control plots. DCD was then applied evenly over the urine plus DCD and to the DCD-only treatment plots using a spray gun. After treatment application, 2 L of water was applied to all plots to wash the leaves and allow the treatments to infiltrate into the soil.

Figure 3.8 Prior to treatment application gas and soil sampling rings were installed into each of the plots, using a board and a sledge hammer to push the rings evenly into the soil.

Figure 3.9 Applying the urine treatment (left) and the DCD (right) to the plots.

3.2.7 Soil sampling

Two soil cores were randomly taken from within each soil sampling plot for KCl extractions, DNA extractions, pH readings and soil moisture. These were taken weekly before the treatment applications, and for the first month following the Urine/DCD applications, then fortnightly thereafter. Holes remaining from cores were filled with topsoil and then marked to ensure they weren't resampled. Soil cores were placed in a plastic zip lock bag, broken up and well mixed before subsamples were taken. Methods used for ammonium, nitrate and DCD extractions are described in 3.1.5 and 3.1.6. The remaining soil was stored at -20^{0}C.

3.2.8 Nitrous oxide measurement

Nitrous oxide emissions were sampled using a modified closed chamber method from Hutchinson and Mosier (1981) (Figure 3.10). All gas sampling rings contained a trough around the perimeter, which prior to sampling was filled with water to provide a seal. Gas chambers were then lined up next to the chambers ready for sampling. At time zero, the gas chamber was placed over the soil ring and in the trough to seal the chamber. The 'a' vial (12 ml) was then placed on the gas sample needle and the syringe needle inserted into the vial (Figure 3.11). The syringe was drawn to 60 mL of chamber air and discarded. This was done once for each vial to 'rinse' the vial with chamber air before a subsample was taken. Once the air was discarded, approximately 25 mL of chamber air was drawn. The chamber needle tap was then closed and removed from the vial. The 25 mL of sample was pumped into the vial, the vial removed, and the remaining air pumped out of the syringe. At the next 1 minute interval the next chamber was started. For the field trial, samples were collected in groups of 16 which allowed four minutes before the next samples were collected. At 20 min (b vial) and 40 min (c vial) samples were collected similarly to the initial (a) sample but after the 'rinse' step the syringe was drawn to 60 mL of chamber air, three times. This allowed for the air in the chamber to be mixed before a 25 mL subsample was taken. At the end of sampling the chambers were removed and stored on site to prevent damage to the needle set up on the chambers.

Gas sampling started on day 1 (10th August 2012) and occurred twice a week for the duration of the study. N_2O was measured using a gas chromatograph (SRO8610 linked to a Filson 222XL autosampler) using an Electron Capture Detector (ECD) (SRI Instruments, USA) and quantified using stored ambient air samples. Elevated concentrations were achieved using ethylene and acetylene in stored air samples.

Figure 3.10 Gas rings with water filled trough (left) and gas chamber with rubber septum and white pressure cap (right). The gas ring was left permanently in the field while the gas chamber was only used while sampling.

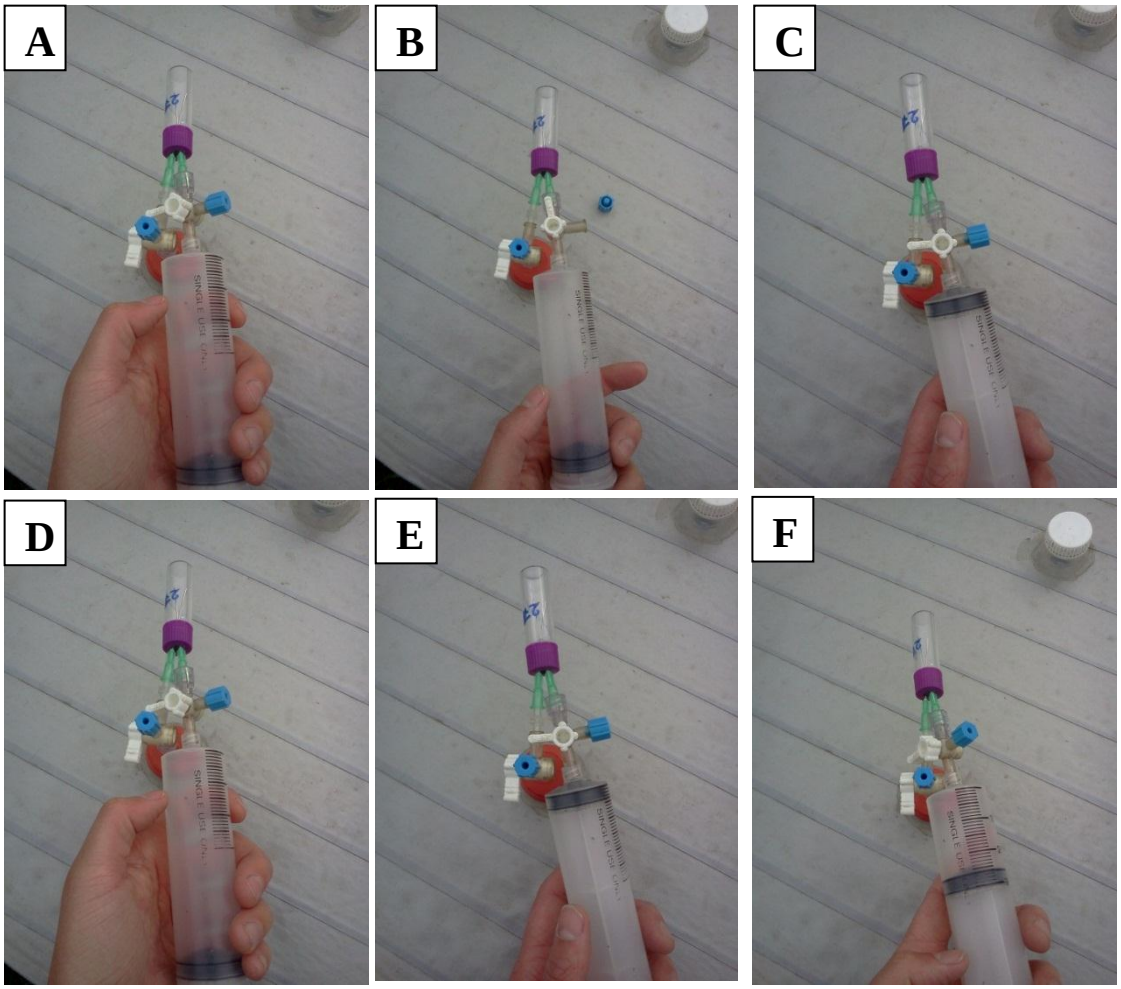

Figure 3.11 Gas sampling method used in the field. 60 mL of gas was drawn into the syringe (A), the tap closed, the stopper removed and the air pushed out to 'rinse' the vial (B), the stopper was then replaced. For the initial sample 25 mL is drawn pumped into the vial (F), for time 20 min and 40 min, 60 mL of air drawn and pumped back into the chamber three times (D and E).

3.2.9 AOA/AOB assays

Samples were collected at day 1, 7, 14, 30, 45 and 90 and stored at -80˚C until DNA extraction. DNA extraction methods are described in section 3.1.7.1. To measure *amoA* gene abundance for AOB and AOA, real time qPCR was carried out as described in section 3.1.7.2.

Chapter 4

Effect of soil aggregate size on N_2O emissions and ammonia oxidising communities – an incubation study

4.1 Introduction

Levels of atmospheric nitrous oxide (N_2O) have increased since the industrial era (Choudhary et al. 2002) with the main anthropogenic source of N_2O identified as agricultural soils. N_2O is a potent greenhouse gas known for its high global warming potential and ability to destroy ozone (Intergovernmental Panel on Climate Change 2007b). In New Zealand, agriculture accounts for 96% of the total N_2O emitted. This is a function of the year round pastoral farming resulting in high rates of inorganic nitrogen (N) to be deposited through animal excreta (Clough et al. 2004).

Soil N_2O production is influenced by a range of microbiological, chemical and physical soil processes and properties (Diba et al. 2011). The majority of N_2O is produced biologically as part of the nitrogen (N) cycle through the production pathways of nitrification and denitrification (Wrage et al. 2001). The first step in the N-cycle, ammonia oxidation, is important for N_2O emissions as it is the rate-limiting step, supplying substrate for denitrification (Di et al. 2010b). Furthermore, nitrification can also account for some N_2O emissions through nitrifier denitrification (Wrage et al. 2001). Denitrification is the main process responsible for N_2O emissions when soil moistures are at or above field capacity and oxygen is limiting.

Soil aggregates are an important controlling factor for soil properties which influence N_2O emissions (Diba et al. 2011). Soil aggregates influence soil pore size distribution which affects aeration, water retention, and drainage. Soil aggregates are defined as "groups of soil particles that are bound to each other more strongly than to adjacent particles" (Diba et al. 2011). It is hypothesised that aggregate size can affect denitrification rates and hence N_2O emissions by providing anaerobic zones in the centre of the aggregate. Larger aggregates can hold more water inside the micropores and, coupled with lower oxygen (O_2) diffusion rates, lowers the soil O_2 concentration, creating anaerobic 'hotspots' within the aggregate (Sexstone et al. 1985; Diba et al. 2011). The inner part of the soil aggregate is more favourable for bacterial life compared to the outer part (Gregorich et al. 1989) and variation in microhabitat

has been shown to alter bacterial populations (Davinic et al. 2012). Greenwood (1975) predicted if the aggregate radius exceeded 9 mm then an anaerobic zone would be present. In contrast, Sexstone et al. (1985) found anaerobic zones in aggregates down to 4 mm. Renault and Stengel (1994) found that small aggregates only become anaerobic when saturated, however larger aggregates tend to have a constant anaerobic centre. Larger anaerobic zones could lead to higher N_2O production from larger soil aggregates, especially if the aggregate could hold more NO_3^- within its micropores (Diba et al. 2011). Diba et al. (2011) found in a volcanic ash soil that larger aggregates produced more N_2O than smaller aggregates when treated with fertiliser and manure. Similarly, Drury et al. (2004) found that N_2O emissions from denitrification increased with increasing aggregate size in an anaerobic incubation study. Khalil et al. (2005) also found higher N_2O production from larger aggregates and suggested that it was caused by a decrease in aeration within the aggregate. In contrast, Uchida et al. (2008) found that smaller aggregates (0-1 mm) produced higher N_2O emissions than larger aggregates when treated with bovine urine, with aggregates >5.6 mm only producing N_2O after significant compaction.

The nitrification inhibitor, dicyandiamide (DCD), has been shown to reduce N_2O emissions from urine patches by 50-70% (Di & Cameron 2002, 2006; de Klein & Eckard 2008; Smith et al. 2008). It is a mitigation method that is becoming widely researched to reduce N losses from agricultural soils. Nitrification inhibitors work by inhibiting the bacterial enzyme (ammonia mono-oxgenase, *amoA*) which converts NH_4^+ to NO_3^-, reducing the substrate available for denitrification (Amberger 1989). This has environmental benefits as it lowers both N_2O emissions and NO_3^- leaching. However, there is some variation in the efficacy of DCD in reducing N_2O emissions in different soils and it is not clear how much of this variation is attributed to differences in soil structure. Furthermore, the effectiveness of DCD in reducing emissions from various soil aggregate sizes has not been reported in the literature.

The objectives of this study were therefore to determine whether aggregate size affects: 1) N_2O emissions, 2) ammonia oxidising bacteria (AOB) and archaea (AOA) abundances and 3) the effectiveness of the nitrification inhibitor, DCD, in reducing N_2O emissions. Three aggregate sizes (1-2, 2-4 and 4-5.6 mm) were used in this incubation study. It was hypothesised that: 1) larger aggregates would produce higher N_2O emissions in accordance to Diba et al. (2011); 2) that a higher abundance of ammonia oxidising microbes would be found in soil comprised of larger aggregates due to higher anaerobic zones and, 3) DCD would

remain effective at reducing N_2O emissions and NO_3^- concentrations under high N concentrations regardless of aggregate size.

4.2 Methods

4.2.1 Trial set up

A laboratory study was set up to determine the effect of aggregate size on N_2O emissions, 2) AOA and AOB communities, and on DCD effectiveness in reducing N_2O emissions.

A Temuka clay loam soil was collected in two separate batches in November 2011 and February 2012 at a depth of 0-10 cm from the field study site at Lincoln University, Christchurch (43˚38'55"S, 172˚ 28'4"E). Soil was sieved using 5.6 mm, 4.0 mm, 2.0 mm and 1.0 mm sieves. Sieves were stacked on top of one another and aggregate sizes separated into 1-2, 2-4 and 4-5.6 mm treatments, referred to as small, medium, and large respectively. Each aggregate treatment was packed separately to a bulk density of 1.0g/cm^3 in 625 mL glass incubation jars and 50 mL centrifuge tubes. Glass jars were used for N_2O sampling and centrifuge tubes were used for destructive soil sampling. Air flow was maintained for the incubation jars through holes in the incubation lids and puncture holes in the parafilm on the centrifuge tubes.

Urine and DCD were applied to each treatment on the 20th of February 2012 using urine collected from dairy cows grazing at the Lincoln University Research Dairy Farm. The treatments were; Control, Urine (700 kg Urine-N/ha), DCD (10 kg DCD/ha), and Urine plus DCD (700 kg urine-N/ha + 10 kg DCD/ha), referred to as "control", "DCD-only", "urine-only" and "urine+DCD", respectively. Treatments were replicated four times. The incubator was set at 10˚C to represent winter conditions when N_2O emissions are highest (Di et al. 2010a). Treatments were placed in the incubator using a randomised block design (Figure 4.1).

4.2.2 Field capacity

To determine the field capacity for each aggregate size, a soil core with a volume of 209.3 cm^3 was packed to a bulk density of 1.0 g/cm^3, and wet up until the soil was saturated. The core was then placed on a tension table at 1 m suction (-1 bar) for 2 days. The cores were then weighed, dried and reweighed to calculate the field capacity (FC = (mass of water

(g)/mass of dry soil (g))*100). Soil moisture was maintained at field capacity throughout the trial by adjusting the weight of each incubation jar and centrifuge tube with deionised water after gas sampling.

1 Large Control	**5** Small DCD	**9** Medium DCD	**13** Large Urine	**17** Large Control	**21** Large DCD	**25** Small DCD	**29** Large Control	**33** Medium Urine	**37** Small DCD+Urine	**41** Large Urine	**45** Small DCD
2 Small Urine	**6** Small DCD+Urine	**10** Small Control	**14** Medium Control	**18** Medium Urine	**22** Small DCD	**26** Large DCD	**30** Medium DCD+Urine	**34** Medium Control	**38** Large DCD+Urine	**42** Small Control	**46** Small Urine
3 Medium Urine	**7** Large DCD	**11** Large DCD+Urine	**15** Small Urine	**19** Medium DCD+Urine	**23** Large DCD+Urine	**27** Small Control	**31** Small DCD+Urine	**35** Small Urine	**39** Large Control	**43** Medium Urine	**47** Medium DCD+Urine
4 Medium Control	**8** Medium DCD+Urine	**12** Large Urine	**16** Small DCD+Urine	**20** Small Control	**24** Medium DCD	**28** Medium DCD	**32** Large DCD+Urine	**36** Large Urine	**40** Medium DCD	**44** Medium Control	**48** Large DCD

----------FRONT OF INCUBATOR----------

Figure 4.1 A randomised block design was used in the incubation study. Treatments were randomly allocated using a random number generator.

4.2.3 Nitrous oxide sampling

Nitrous oxide sampling was carried out using a modified closed chamber method similar to Hutchinson and Mosier (1981). Gas sampling lids contained a septum, three way valve and needle shown in Figure 4.2. Gas samples were taken at time 0 and 30 mins twice weekly from 21st of February 2012 to 15th of May 2012 (Day 84), then once weekly until the end of the experiment. N_2O was measured using a gas chromatograph (SRO8610 linked to a Filson 222XL autosampler) using an Electron Capture Detector (ECD) (SRI Instruments, USA) and quantified using stored ambient air samples. Elevated concentrations were achieved using ethylene and acetylene in stored air samples.

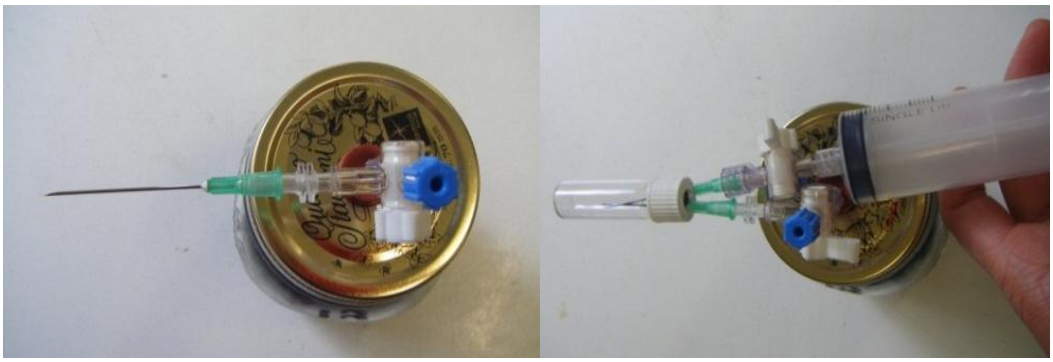

Figure 4.2 Gas sampling lid containing a septum, needle and three-way valve for N_2O sampling. To draw a sample, a 60 mL syringe was used.

4.2.4 Soil sampling and analysis

For each soil sampling date, two racks containing 48 centrifuge tubes (Figure 4.3) were removed from the incubator, upended into a plastic bag and well mixed. Subsamples were taken from each replicate to determine concentrations of DCD, NO_3^- and soil moisture.

DCD was extracted from 5 g of soil using 25 mL of deionised water. Samples were shaken for 1 hour and centrifuged for 20 mins and then filtered using Whatman No. 41 filter paper. DCD concentration was analysed on a Shimadzu series High Performance Liquid Chromatography (Tokyo, Japan) using a cation-H guard column (Phenomenex, USA) and a 0.025 M sulphuric acid mobile phase at a flow rate of 0.6 ml/min, by UV detection at a wavelength of 210 nm.

Figure 4.3: Gas sampling jars with incubation lids (right), and centrifuge tubes for destructive soil sampling (left), total 48 tubes for each soil sampling date.

Extraction of NO_3^- was carried out with the addition of 25 mL of 2 M KCl to 5 g of soil. Samples were shaken for 1 hour and centrifuged for 10 mins and the supernatant filtered using Whatman No. 41 filter paper. Nitrate concentrations were analysed using a flow injection analyser (FIA) (FOSS FIA star 5000 triple channel analyser) with SoFIA software version 1.30 (Foss Tecator AB, Sweden).

Soil moisture was determined for each of the 48 samples at each sampling date by weighing a subsample of soil (approximately 10 g), drying it at 105˚C for 24 hours and then reweighing it. Soil moisture was calculated using the following formula: ((wet soil (g) - dry soil (g))/dry soil (g)) x 100.

4.2.5 AOB and AOA assays

Subsamples were taken at Day 1, 7, 28, 70 and 84 after the application of treatments to determine ammonia mono-oxygenase (*amoA*) gene copy numbers of AOB and AOA. Soil samples were stored at -80˚C prior to extraction.

DNA was extracted from frozen soil (0.25 g) using NucleoSpin® Soil Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. DNA was eluted with 100µL of Buffer SE (Macherey-Nagel, Düren, Germany LOT. PAF00456026) and stored at -20˚C before analysed.

Polymerase chain reaction's (PCR) were set up using the CAS1200 Robotic liquid handling system (Corbett Life Science, Australia), and real-time PCR was performed on a Rotor-Gene™ 6000 (Corbett Life Science). 10-fold dilutions of template DNA was used for the PCR. Bacterial and archaeal *amoA* genes were quantified using the primers amoA1F/amoA2R (Rotthauwe et al. 1997) and Arch-amoAF/Arch-amoAR (Francis et al. 2005) respectively, with SYBR® Premix Ex Taq™ (TaKaRa, Japan) using the thermal profiles as described in Di et al. (2009). The 20 µL reaction mixture contained 10 µL of SYBR® Premix Ex Taq™ including primers, and 1.5 µL of template DNA. To confirm PCR product specificity, a melting curve analysis was carried out, by measuring fluorescence continuously as the temperature was increased from 50 to 99˚C. Data analysis was carried out using Rotor-Gene™ 6000 series software 1.7.

Standard curves for real-time PCR assays were developed using the following method. Briefly, the bacterial and archaeal *amoA* genes were PCR amplified from the extracted DNA with the aforementioned primers. The PCR products were purified using the PCR clean-up kit (Axygen) and cloned into the pGEM-T Easy Vector (Promega, Madison, WI) and the resulting ligation mix transformed into *Escherichia coli* JM109 competent cells (Promega) following the manufacturer's instructions. Plasmids which were used as standards for quantitative analyses were extracted from the correct insert cloners from each target gene and sent for sequencing. A Qubit™ fluorometer (Invitrogen NZ) was used to determine the plasmid DNA concentration and the copy numbers of target genes calculated. Tenfold serial dilutions of the known copy number of the plasmid DNA were then subjected to a real-time PCR assay in triplicate to generate an external standard curve and to ensure amplification efficiency.

4.2.6 Statistical analysis

Mean values and standard errors of the means for NO_3^- concentrations, DCD concentrations and N_2O emissions were calculated based on the four replicates for each treatment using Microsoft Excel 2010 (Microsoft Corporation, USA). Least significant differences and p-values were calculated following analysis of variance using Genstat© (Version 15.1, VSN International Ltd, U.K.).

4.3 Results

4.3.1 N_2O emissions

Aggregate size, overall, had a significant effect on cumulative N_2O emissions from the sieved Temuka clay loam soil up to both days 51 and 288 (main effect linear trend p-values = 0.003 and 0.040 respectively; Appendix 2). Temporally, for the "urine-only" treatments, the medium and large aggregates followed similar N_2O fluxes (Figure 4.4). In contrast, the flux of N_2O for the small aggregates was different. The medium and large aggregates peaked at day 35 with emission peaks of 0.233 mg N_2O-N/m^2/hr. and 0.254 mg N_2O-N/m^2/hr. respectively, which was approximately 1.5 times higher than the emission peak of the small aggregates at 0.161 mg N_2O-N/m^2/hr. on day 21. After day 66, small aggregates continued to produce higher N_2O emissions than large and medium aggregates. This continued higher N_2O emission rate led to the final total N_2O emissions for each aggregate size being the same. Thus, although temporally the N_2O flux was significantly different between aggregate sizes, the total N_2O emitted was not significantly different (Figure 4.4B, Table 4.1). This is seen in the cumulative N_2O emissions for the "urine-only" treatments, where by day 51 large aggregates had produced 1541 g N_2O-N/ha, which were significantly ($p<0.05$) higher emissions than 855 g N_2O-N/ha, produced by the small aggregates (Table 4.1). However, at day 288 (total emissions) there is no significant difference between aggregate sizes, with total emissions ranging from 3908- 4046 g N_2O-N/ha.

In all aggregate sizes, the addition of DCD to the urine treatments significantly ($p<0.05$) reduced total N_2O emissions compared to urine-only treatments (Table 4.1) by 72%, 79% and 85% for the large, medium and small aggregates respectively. In the urine+DCD treatment, by day 51 and 297, small aggregates had significantly lower cumulative N_2O emissions compared to medium and large aggregates (Table 4.1). The addition of DCD alone did not reduce N_2O emissions from the control treatment and there was no significant difference between aggregate sizes in the DCD-only treatment (Figure 4.4B).

Table 4.1 Cumulative N_2O emissions treatment mean table for up to day 51 and 288 (BT = back transformed mean values (g N_2O-N/ha equivalent).

	Day 51							
Treatment	Control		DCD-only		Urine-only		Urine + DCD	
Aggregate size	**Log_{10} mean**	**(BT mean)**	**Log_{10} mean**	**(BT mean)**	**Log_{10} mean**	**(BT mean)**	**Log_{10} mean**	**(BT mean)**
Large	1.724	(53)	1.741	(55)	3.187	(1538)	2.928	(847)
Medium	1.722	(53)	1.717	(52)	3.150	(1412)	2.833	(681)
Small	1.776	(60)	1.680	(48)	2.932	(855)	2.567	(369)
LSD (5%)	0.196							
	Day 288							
Large	2.345	(221)	2.374	(237)	3.592	(3908)	3.030	(1072)
Medium	2.415	(260)	2.421	(264)	3.619	(4159)	2.951	(893)
Small	2.352	(225)	2.316	(207)	3.607	(4046)	2.785	(610)
LSD (5%)	0.134							

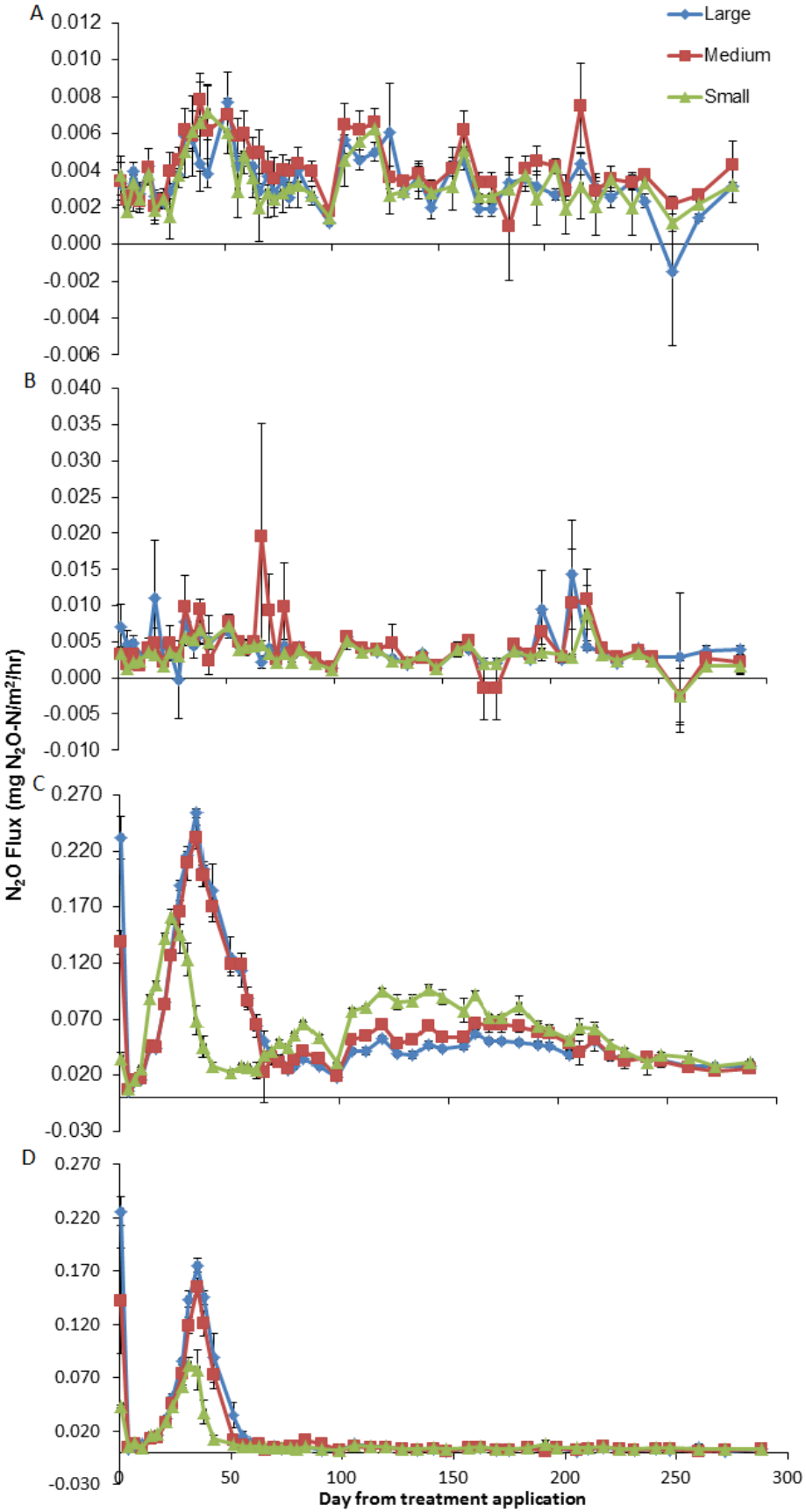

Figure 4.4 N_2O flux for large, medium and small aggregate sizes; (A): control; (B): DCD-only; (C): urine-only; and (D) urine+DCD from the incubation study. Error bars show standard error of the mean (SEM).

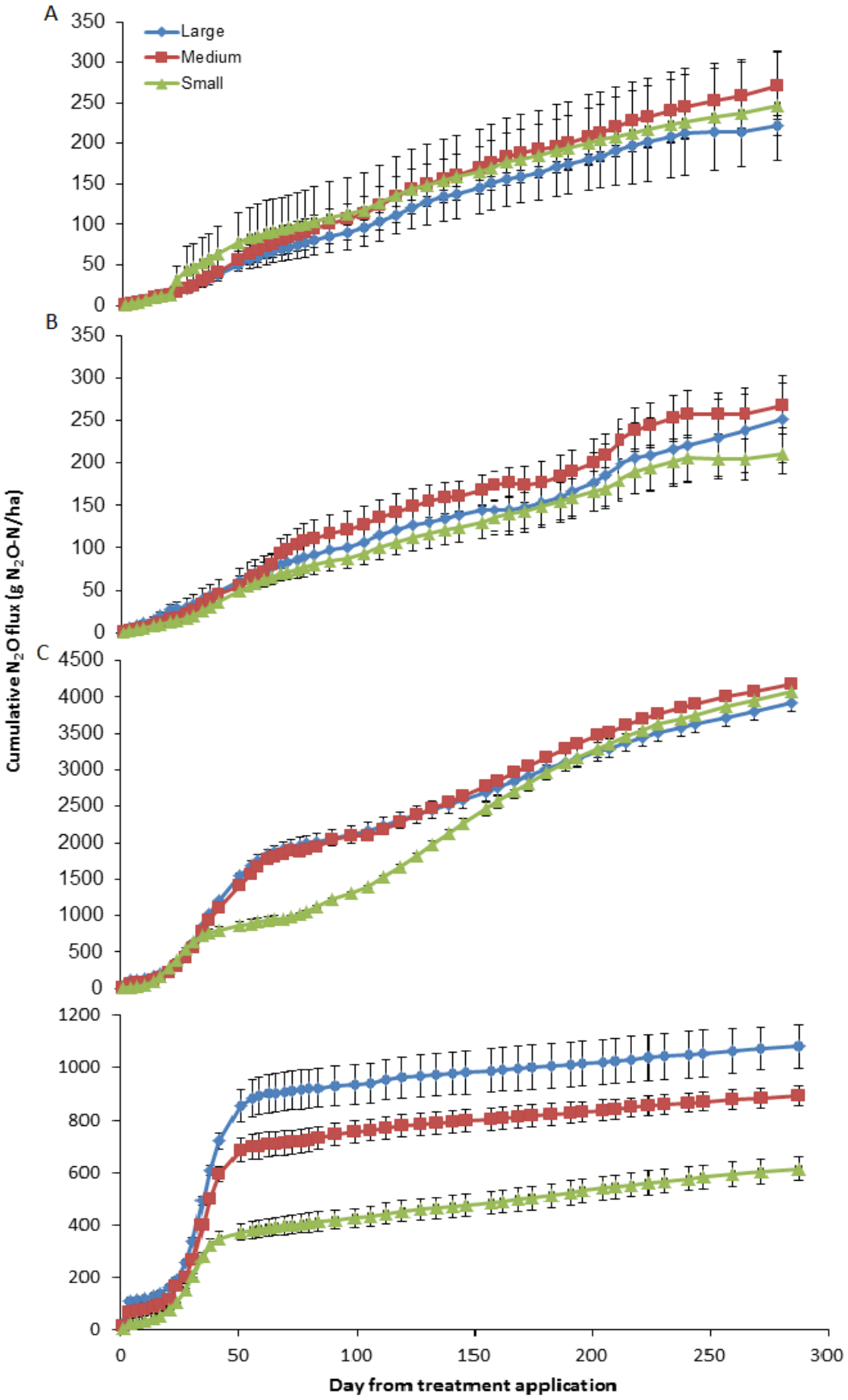

Figure 4.5 cumulative N2O flux for large, medium and small aggregate sizes; (A): control; (B): DCD-only; (C): urine-only; and (D): urine+DCD for incubation study. Error bars indicate SEM.

4.3.2 Soil NO_3^- concentrations

Soil NO_3^- concentrations increased rapidly from day 1 to day 84 in all treatments (Figure 4.6**)**. The increase in NO_3^- concentrations was seen in the urine-only treatments with average increases from, 8.5 mg NO_3-N/kg of dry soil at day 1 to 401 mg NO_3-N/kg of dry soil at day 84. At both day 84 and day 297, urine-only treatments had significantly ($p<0.05$) higher NO_3^- concentrations compared to the control (Table 4.2). Furthermore, medium aggregates had significantly ($p<0.05$) higher soil NO_3^- concentrations than small and large aggregates. At day 84, medium aggregates had NO_3^- concentrations of 445 mg NO_3-N/kg of dry soil, whereas, large and small had 402 and 355 mg NO_3-N/kg of dry soil, respectively. The addition of DCD significantly ($p<0.05$) lowered NO_3^- concentrations compared to the "urine-only" treatment with the largest reduction being from 722 mg NO_3-N/kg of dry soil to 208 mg NO_3-N/kg of dry soil seen in the medium aggregates at day 297 (Table 4.2).

When NO_3^- concentration was averaged over the 297 day study period, there was a significant difference ($p<0.05$) between aggregate sizes (Figure 4.7) Medium aggregates accumulated 11.3 mg NO_3-N/kg of dry soil per day, which was significantly higher than 10.5 and 9.5 mg NO_3-N/kg dry soil per day seen in the large and small aggregates respectively. The addition of DCD reduced the average NO_3^- accumulation per day for all soil aggregates from 10.44 mg NO_3-N/kg/day to 3.02 mg NO_3-N/kg/day, an average reduction of 71%.

Table 4.2 Treatment mean table for NO_3^- concentrations (mg/kg of dry soil) at day 84 and 297. Day 84 and 297 were chosen as NO_3^- concentration peaked on these days.

	Day 84							
Treatment	Control		DCD-only		Urine-only		Urine + DCD	
Aggregate size	**Log_{10} mean**	**(BT mean)**	**Log_{10} mean**	**(BT mean)**	**Log_{10} mean**	**(BT mean)**	**Log_{10} mean**	**(BT mean)**
Large	1.364	(23)	1.283	(19)	2.604	(402)	2.022	(105)
Medium	1.470	(30)	1.452	(28)	2.648	(445)	2.013	(103)
Small	1.460	(29)	1.435	(27)	2.550	(355)	2.093	(124)
LSD (5%)	0.091							
	Day 297							
Large	1.975	(94)	1.948	(89)	2.789	(615)	2.274	(188)
Medium	2.054	(113)	1.960	(91)	2.859	(722)	2.319	(208)
Small	2.034	(108)	1.901	(80)	2.760	(575)	2.361	(230)
LSD (5%)	0.013							

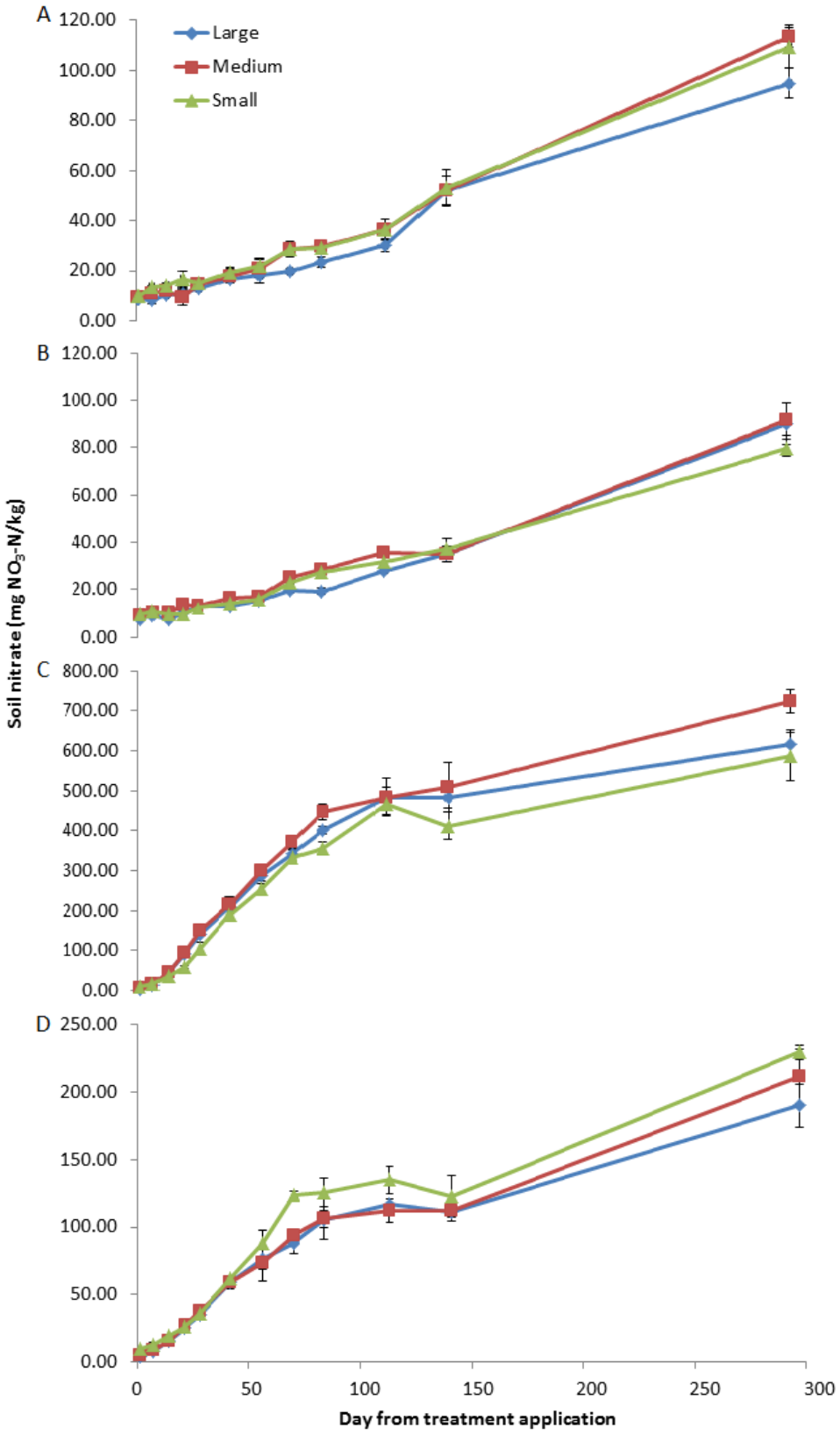

Figure 4.6 Soil nitrate concentration for large, medium and small aggregate sizes; (A): control; (B): DCD-only; (C): urine-only; and (D): urine+DCD. Error bars indicate SEM.

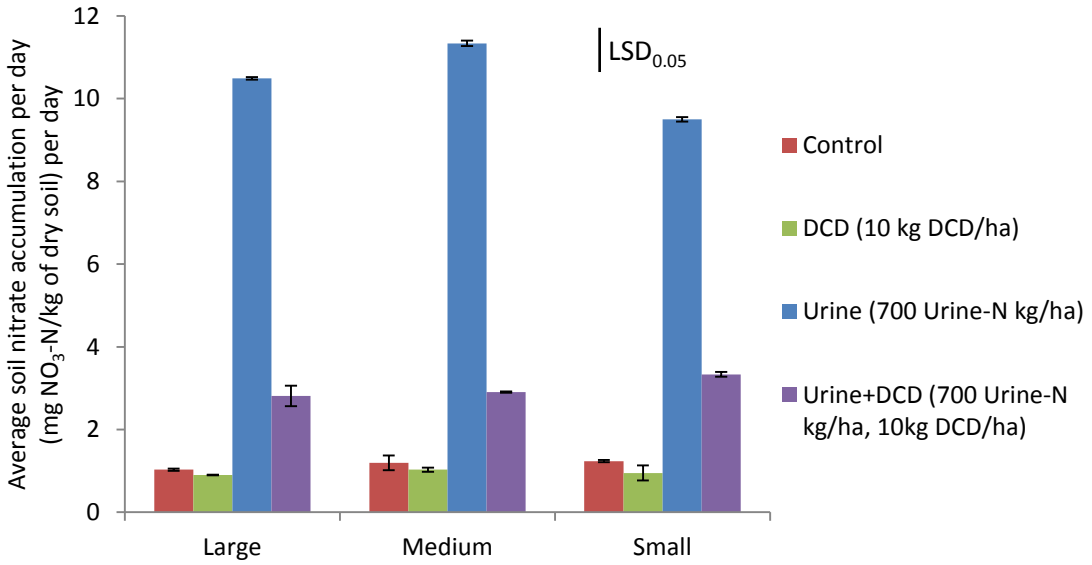

Figure 4.7 Average soil NO_3^--N concentration accumulation per day for large, medium and small aggregate sizes in the incubation trial. Error bars indicate SEM. $LSD_{0.05}$ demonstrates the least significant difference ($P<0.05$).

4.3.3 DCD concentration

The concentration of DCD did not vary significantly between aggregate sizes at each sampling date and degraded at a similar rate in all DCD treatments. When DCD concentration was averaged across all DCD-only and urine+DCD treatments, the DCD degraded at an exponential rate (Figure 4.8) with a half-life of 87 days.

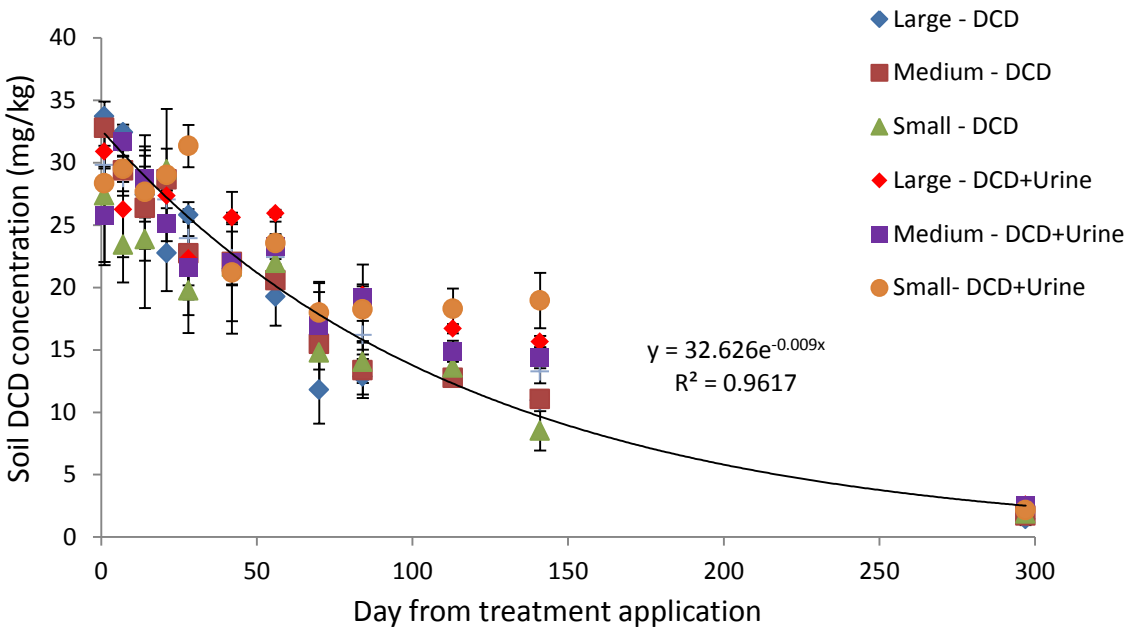

Figure 4.8 DCD concentration in DCD-only and urine+DCD treatments for large, medium and small aggregate sizes. Trend line shows exponential trend for averaged data across all DCD-only and urine+DCD treatments at each sampling date. Error bars indicate SEM.

4.3.4 Ammonia oxidising community abundance

4.3.4.1 Ammonia oxidising bacteria

In the control treatment, aggregate size did not significantly affect ammonia oxidising bacteria (AOB) *amoA* gene abundance (Figure 4.10). However, with the addition of urine, the AOB *amoA* gene abundance significantly ($p<0.05$) increased compared to the control treatment in all aggregate sizes (Figure 4.9, Figure 4.10). The largest increase was seen in the small aggregate size, with AOB abundance significantly increasing from $4.74x10^7$ copy numbers/g of soil in the control to $2.36x10^8$ copy numbers/g of soil in the urine-only treatment (Figure 4.10). The abundance peak in the urine-only treatment for AOB *amoA* gene abundance was seen at day 28 for the medium aggregates and day 70 for the large and small aggregates (Figure 4.10).

The addition of DCD to the urine-only treatment significantly ($p<0.05$) decreased the AOB *amoA* gene abundance compared to the urine-only treatment in all aggregate sizes, with the largest reduction in the small aggregate size. The application of DCD in the absence of urine, did not significantly affect AOB *amoA* gene abundance compared to the control (Figure 4.9, Figure 4.10).

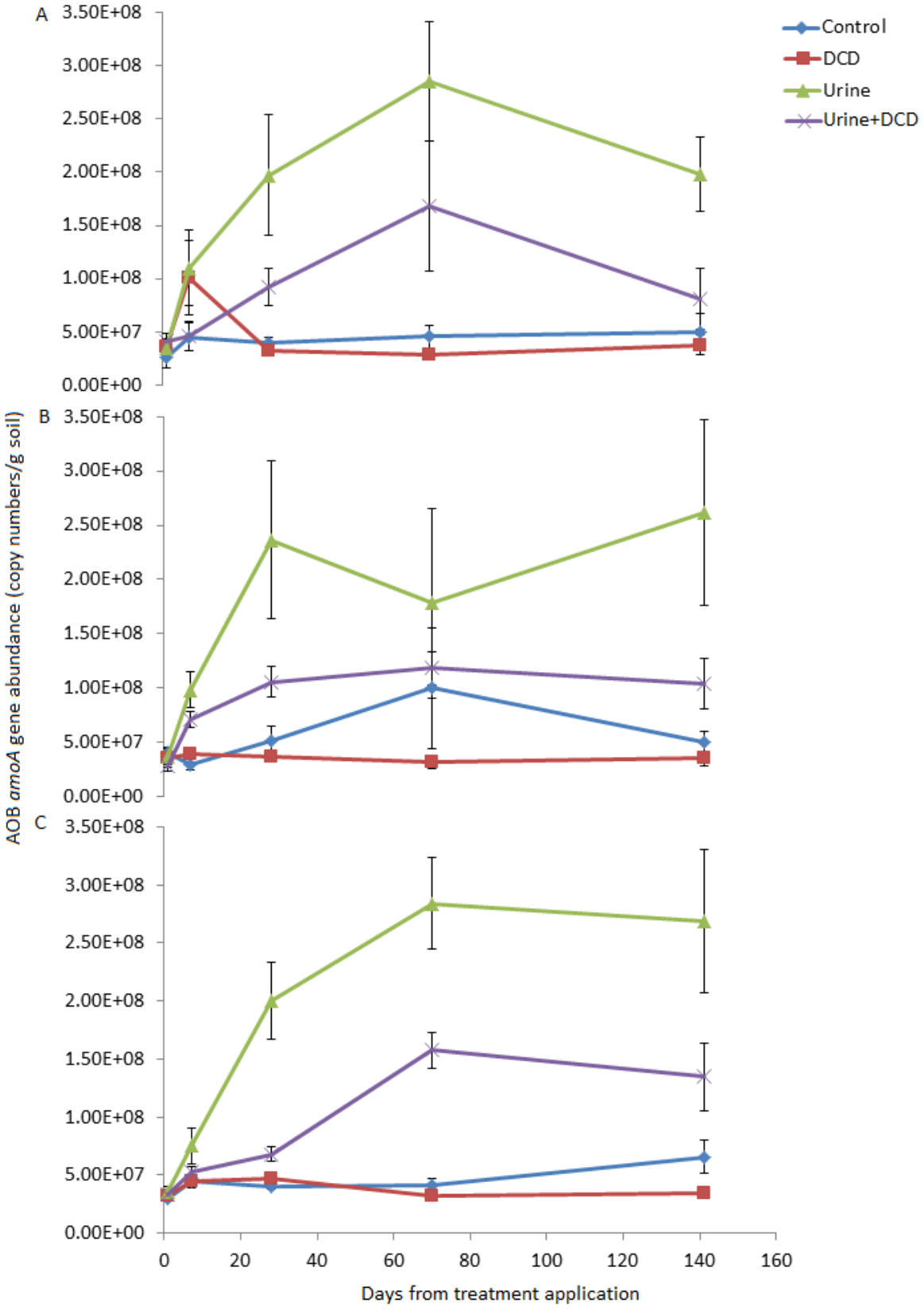

Figure 4.9 AOB amoA gene abundance for (A): Large aggregates; (B): Medium aggregates; and (C): Small aggregates. Error bars indicate SEM.

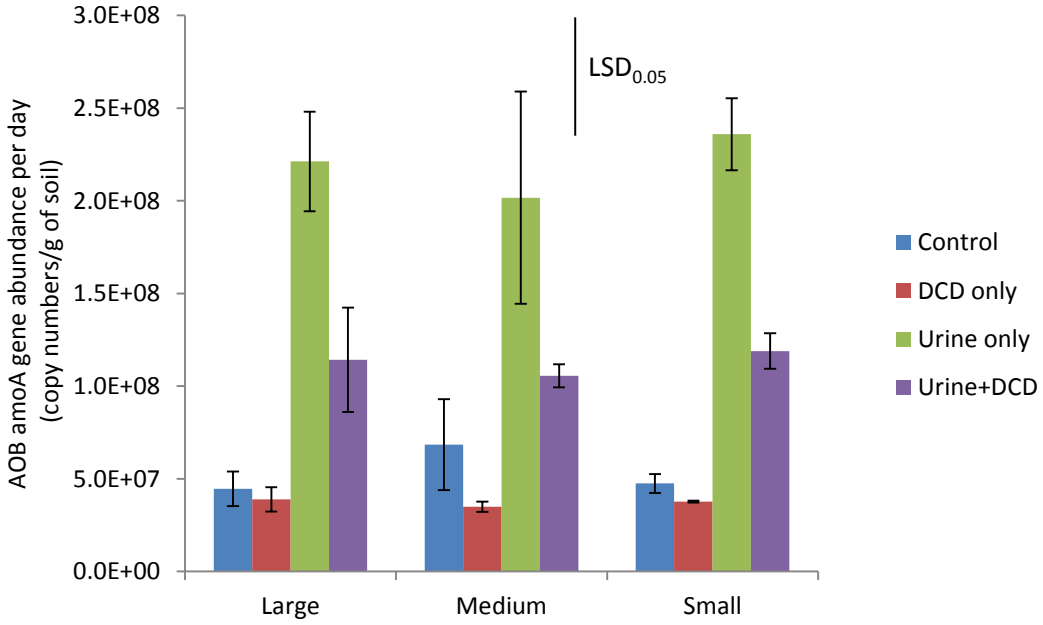

Figure 4.10 Weighted average for AOB amoA gene abundance (*amoA* copy numbers/g of soil/ day). Error bars indicate SEM and LSD indicates least signficant difference (p = 0.05).

4.3.4.2 Ammonia oxidising archaea

Aggregate size did not affect *amoA* gene abundance of ammonia oxidising archaea (AOA), with non-significant differences seen in the control, DCD-only and urine-only treatments (Figure 4.11, Figure 4.12, Table 4.4). AOA *amoA* gene abundances were lower than AOB *amoA* gene abundances in all treatments.

The addition of urine significantly ($p<0.05$) decreased the AOA *amoA* gene abundance compared to the control, in the large aggregate size only (Figure 4.12). However, in the medium and small aggregates there was no significant effect of urine on AOA *amoA* gene abundance.

In the urine+DCD treatment, the application of DCD had no significant effect on AOA *amoA* gene abundance compared to the urine-only treatment (Figure 4.11, Figure 4.12).

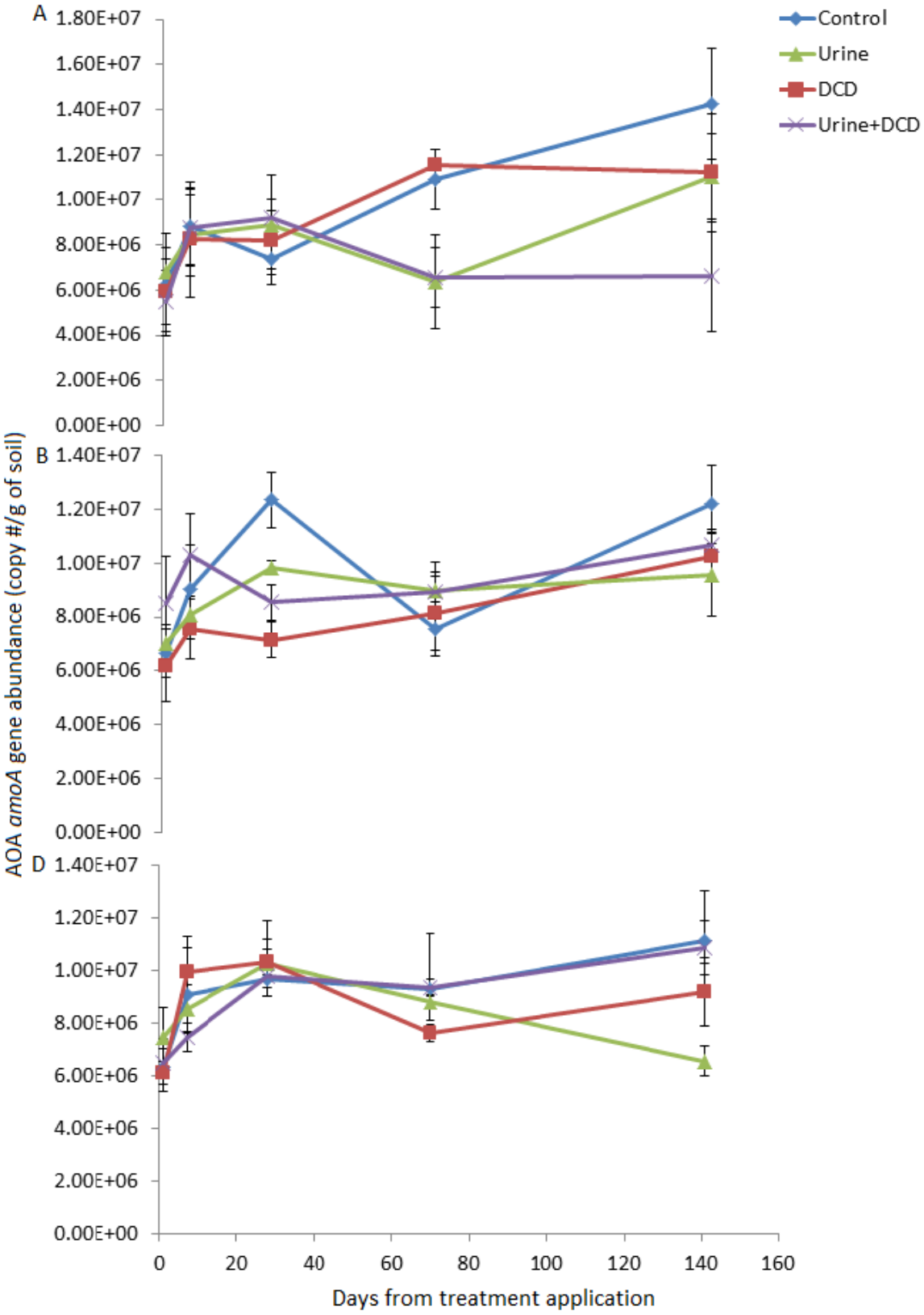

Figure 4.11 AOA amoA gene abundance for (A): Large aggregates; (B): Medium aggregates; and (C): Small aggregates. Error bars indicate SEM.

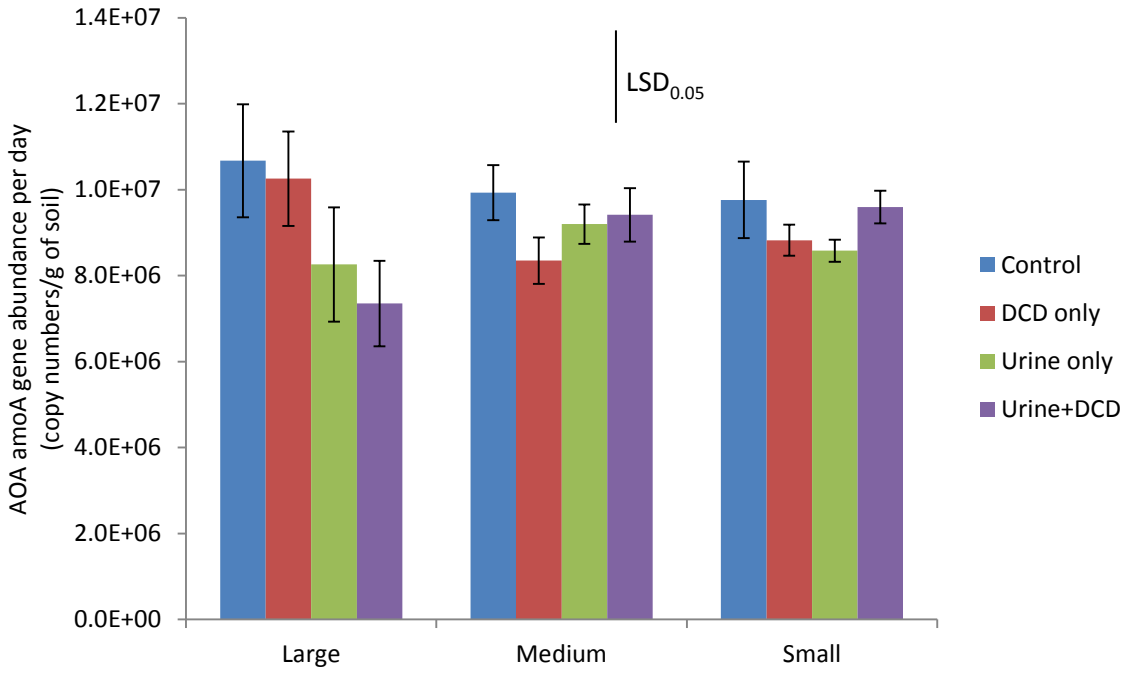

Figure 4.12 Weighted average for AOA amoA gene abundance (amoA copy numbers/g of soil/ day). Error bars indicate SEM and LSD indicates least signficant difference (p = 0.05).

4.4 Discussion

4.4.1 Nitrous oxide and aggregate size

With urine addition, large (4-5.6 mm) and medium (2-4 mm) aggregates were found to have a significantly ($p<0.05$) higher N_2O emission peak than small (1-2 mm) aggregates. N_2O is produced in partial anaerobic conditions through the processes of nitrification and denitrification as defined in Wrage et al. (2001). Anaerobic "hotspots" found in the centre of soil aggregates have been hypothesised as being an area of high localised N_2O production. Large soil aggregates have relatively low O_2 diffusivity, producing larger anaerobic centres and thus higher N_2O emissions than small aggregates. Greenwood (1975) stated that anaerobic centres would not be found in aggregates with a radius less than 9 mm. Sexstone et al. (1985) found aggregates greater than 10 mm had measurable anaerobic centres. However, they also found small anaerobic centres in aggregates down to 4 mm. In this study, the high N_2O peak seen in the large and medium aggregates is in agreement with Sexstone et al. (1985), demonstrating that anaerobic centres may be present in aggregates as small as 4mm. With urine addition, NO_3^- accumulation/day was highest in the medium and large aggregates. This suggests that larger aggregates accumulated more NO_3^-, and therefore have more substrate available for denitrification to occur within the anaerobic zone, thus producing the greater N_2O emissions. This is similar to Diba et al. (2011) who also found that high N_2O fluxes in large aggregates was associated with greater amounts of NO_3^-.

In addition to higher NO_3^- accumulation, high N_2O peaks in the medium and large aggregates could have been caused by an increase in readily mineralisable C found in larger soil aggregates. Gregorich et al. (1989) states that macro-aggregates with a radius greater than 2.5mm have more readily mineralisable organic matter, whereas micro aggregates less than 2.5mm have protected organic matter which only become available for biodegradation when disturbed. As soil micro-organisms get energy from organic materials, and N_2O formation is a microbial process, N_2O production can be linked to the availability of organic C. Diba et al. (2011) found a positive relationship between N_2O and CO_2 and suggested that N_2O production was strongly affected by organic matter decomposition. However, this can only be inferred in this study as organic C concentration was not measured.

By day 66, N_2O emissions from the medium and large aggregates amended with urine were less than the small aggregates amended with urine. This "switch" at day 66 caused the total N_2O emitted over the trial period to be the same for all aggregate sizes. Thus, although the

N_2O flux was temporally different, overall the total amount of N_2O emitted was the same. However, this cannot be linked to the availability of NO_3^-, as NO_3^- concentrations throughout the trial period were similar for the various aggregate sizes, and there was no "switch" in concentrations at day 66. Furthermore, this cannot be caused by difference in ammonia oxidising communities as there was no significant difference between aggregate sizes for AOB and AOA *amoA* gene abundance at day 70. Uchida et al. (2008) found that over time aggregates treated with urine became unstable, especially in the smaller aggregates (<2 mm). The high salt content of urine or the increase in pH following urine addition can cause partial dispersion of aggregates (Uchida et al. 2008). As small aggregates have a high surface area to volume ratio they could be more susceptible to chemical disruption by urine addition. Disruption of soil aggregates can cause previously unavailable carbon to be released into soil solution and this can be readily used by microbes (Gregorich et al. 1989). Lambie et al. (2012) found 10 times more carbon solubilisation in soil treated with urine compared to water. Uchida et al. (2008) found increased instability in small aggregates at the end of their trial (day 62). Theoretically, in this trial, similar instabilities could have been found with urine addition in the small aggregates by day 66. The instability of the small aggregates could have increased the level of potential mineralisable carbon over time, increasing denitrifier activity and hence N_2O emissions after day 66.

Medium and large aggregate sizes had similar N_2O flux rates. Similarly, Uchida et al. (2008) found that aggregate sizes 2-4 mm and 4-5.6 mm (equivalent to medium and large aggregates respectively in this study) followed a similar N_2O flux. This lack of difference could be attributed to the small difference in aggregate size causing the anaerobic centres to be similar within the large and medium aggregate treatments.

4.4.2 Ammonia oxidising communities

Overall, aggregate size had no significant effect on AOB and AOA *amoA* gene abundance. However, with the addition of urine *amoA* gene abundance significantly ($p < 0.05$) increased in all aggregate sizes compared to the control, with the highest increase in the small aggregates. The greater AOB abundance increase in the small aggregates could be caused by a higher surface area to volume ratio, allowing the AOB to have greater accessibility to the urine-N substrate. In contrast, AOA *amoA* gene abundance in the large aggregates significantly ($p < 0.05$) decreased with the addition of urine but there was no significant reduction in AOA *amoA* gene abundance in the medium and small aggregates. The significant

AOA *amoA* gene abundance reduction in the large aggregates could be caused by larger aggregates holding more NO_3^- within the aggregate's micropore (Diba et al. 2011), therefore significantly inhibiting the AOA within the aggregate compared to the medium and small aggregates. The increase in AOB *amoA* gene abundance demonstrates that AOB growth is favoured in high N conditions. This is similar to the findings of Di *et al*. (2010b) and supports the hypothesis that AOB prefer high-N environments whereas AOA prefer low nutrient environments (Erguder et al. 2009; Di et al. 2010a).

The addition of DCD significantly reduced AOB *amoA* gene abundance (Figures 4.10, 4.12). However, DCD did not reduce AOA *amoA* gene abundance. This is in agreement with previous findings of Di et al. (2009) who found the addition of DCD decreased AOB *amoA* gene abundance and that AOA *amoA* gene abundance was unaffected. This was probably due to inhibition of the AOA communities by the urine-N supplied. Similarly, the application of DCD in the absence of urine had no significant effect on AOB or AOA *amoA* gene abundance because these microbial communities do not grow in the absence of NH_4^+ substrate stimulation.

Ammonia oxidising archaea *amoA* gene abundance was lower than AOB *amoA* gene abundance in all treatments. This is in accordance with the findings of Di et al. (2009) where AOB abundance was higher than AOA abundance in the Canterbury soil.

4.4.3 DCD effectiveness

DCD was effective at reducing N_2O emissions and NO_3^- concentrations from urine treatments in all soil aggregate sizes. DCD significantly (0.05) reduced total N_2O emissions from urine amended treatments by 72%, 79% and 85% in the large, medium and small aggregates respectively. This is similar to other studies where reported reductions in N_2O emissions following DCD applications ranged between 60-90% (Di & Cameron 2002, 2006; Smith et al. 2008; de Klein et al. 2011). Significantly less N_2O was emitted from the small aggregates amended with urine and DCD compared to the large aggregates amended with urine and DCD. The lower per cent reductions in large aggregates could be caused by the lower surface area to volume ratio of the aggregates causing less or slower diffusivity of DCD into the aggregates. Furthermore, larger aggregates may have the ability to hold the NO_3^- within the micropores of the aggregate (Diba et al. 2011) thus increasing N_2O emissions in the aggregates anaerobic zone compared to the small aggregates, even when amended with DCD.

The reduction in the accumulated NO_3^- was not significant between aggregate sizes and the average reduction with DCD application was 71%. This is higher than the 60% reported from the lysimeter studies in Di and Cameron (2002, 2005). The higher reduction in NO_3^- concentrations in this incubation study may have been due to the separation of the soil into aggregate sizes, creating higher surface areas for DCD to penetrate the soil aggregates.

For this trial, DCD had a half-life of approximately 87 days at 10^0C. This is similar to that reported by Kelliher et al. (2008) where DCD had a half-life at 10^0C of 72+/- 14days and Di and Cameron (2004) who found at 8^0C DCD had a half-life of 111-116 days.

4.5 Conclusions

Aggregate size overall did not affect the total N_2O emitted from the Temuka clay loam soil. However, the N_2O flux temporally varied with aggregate size. With urine addition, medium and large aggregates followed a similar emission profile with a significantly higher peak at day 35 compared to small aggregates, and decreased emissions at day 66. In contrast, in small aggregates the N_2O peak was earlier, at day 21, and 1.5 times smaller than the medium and large aggregates. Furthermore, after day 66 the small aggregates had higher N_2O emissions than the large and medium aggregates. This switch at day 66 caused total N_2O emissions to be the same for all aggregate sizes at the conclusion of the trial.

The initial peak in N_2O emissions supports the hypothesis that large aggregates would have higher N_2O emissions; however, overall the hypothesis has to be rejected because total N_2O emissions are not significantly different between the aggregate sizes. For the small aggregates with urine addition, the continued higher N_2O emissions after day 66 could be caused by the release of labile organic carbon through aggregate disruption (Gregorich et al. 1989) with urine addition (Lambie et al. 2012), which was also reported by Uchida et al. (2008). Additionally, the high surface area to volume ratio of small aggregates means that they are more susceptible to chemical disruption and degradation than larger aggregate sizes.

AOB and AOA *amoA* gene abundance was not affected by aggregate size thus the hypothesis that ammonia oxidising microbial abundance would be greater in large aggregates is rejected. The addition of urine did increase AOB *amoA* gene abundance as also found by Di et al. (2010b). However, AOA *amoA* gene abundance decreased with urine addition in the large aggregates. This is thought to be caused by the large aggregates ability to hold greater amounts of NO_3^- within their micropores (Diba et al. 2011). Furthermore, in agreement with Di et al. (2009) the application of DCD significantly reduced AOB *amoA* gene abundance and did not affect AOA *amoA* abundance.

DCD was effective at reducing N_2O emissions and NO_3^- concentrations in all soil aggregate sizes supporting the hypothesis that DCD would remain effective regardless of soil aggregate size. DCD was more effective in the small aggregate size compared to the large aggregate size. This could have been caused by the higher surface area to volume ratio, allowing greater amounts of DCD to be absorbed within the smaller aggregates, or the ability of larger aggregates to hold more NO_3^- concentrations within the aggregates anaerobic centre. The

DCD half-life at 10^0C was found to be 87 days in this incubation trial, which is similar to the findings of Di and Cameron (2004) and Kelliher et al. (2008).

Chapter 5
The effect of soil pH on N_2O emissions, ammonia oxidising communities and DCD effectiveness - a Field Study

5.1 Introduction

Nitrous oxide (N_2O) is a potent greenhouse gas due to its high global warming potential and ability to destroy stratospheric ozone (Intergovernmental Panel on Climate Change 2007a). Global N_2O emissions have increased by 20% in the last century (Thomson et al. 2012), with the main anthropogenic source being agricultural soils. The majority of N_2O is produced by microbial denitrification as part of the N cycle when the soil is partially anaerobic. However, nitrification can also produce N_2O as a reductive side reaction when soils become waterlogged. Denitrification is an anaerobic process whereby the nitrate (NO_3^-) produced from nitrification is converted to di-nitrogen (N_2) through the obligatory intermediate N_2O. Incomplete denitrification causes a higher proportion of N_2O to be produced and various soil properties can enhance its formation. The ratio of N_2O:N_2 can be affected by soil pH (Wijler & Delwiche 1954), soil moisture content (Smith et al. 1998), NO_3^- concentration (Blackmer & Bremner 1978), carbon supply (Burford & Bremner 1978) and temperature (Keeney et al. 1979; Smith et al. 1998). These factors either enhance the substrate available for denitrification or cause incomplete denitrification by inhibiting the enzyme (N_2O-reductase) responsible for the conversion of N_2O to N_2.

The pH of a soil affects denitrification by altering denitrification rates (Simek & Cooper 2002) and disrupting the denitrification pathway (Wijler & Delwiche 1954). In acidic soils, denitrification rates are lowered, however, N_2O production is favoured, as N_2O-reductase is inhibited and incomplete denitrification occurs. At a high pH, where N_2O-reductase is not inhibited, complete denitrification occurs and N_2 production is favoured (Wijler & Delwiche 1954). Weislien et al. (2009) found a strong negative correlation between N_2O emissions and pH, with almost 5 times higher N_2O emissions from soils with a pH of 3.7 compared to pH of 5.8. Bakken et al. (2012) found that at pH 6 all of the NO_3^- was emitted as N_2O. Whereas, at pH 8.5, Thomsen et al. (1994) found that the NO_3^- was converted completely to N_2. Clough et al. (2004) found that N_2O emissions were higher in a soil with a pH less than 5.9 at field

capacity. However, when the soil was saturated the emissions were lowest in the pH 4.7 soil, highlighting the importance of soil moisture effects on N_2O emissions.

Ammonia oxidisers are important for denitrification as they are responsible for the rate determining step, converting ammonia (NH_3) to NO_3^-. Ammonia oxidising bacteria (AOB) are predominantly responsible for NH_3 oxidation in soils with a high nitrogen (N) content, such as under urine patches in grazed grassland soil (Di et al. 2009). Recent studies have hypothesised that AOA may dominate NH_3 oxidation in low pH and low nutrient soils due to their adaption to extreme environments (Erguder et al. 2009; Di et al. 2010b). However, the role of AOA in nitrification is yet to be fully understood and AOA could dominate N cycling in some soil conditions (Leininger et al. 2006; Erguder et al. 2009; Di et al. 2010b). Nicol et al. (2008) found that bacterial ammonia monoxygynase (*amoA*) gene abundance decreased with acidity, whereas archaeal *amoA* gene abundance increased. Zhang et al. (2011) suggested that AOA and AOB occupied different niches in acidic soils, with NH_3 oxidation driven by AOA rather than AOB in naturally strongly acidic soils.

The nitrification inhibitor, dicyandiamide (DCD), has been shown to effectively reduce N_2O emissions from agricultural soils (Di & Cameron 2002, 2005; Di et al. 2010a; de Klein et al. 2011). DCD works by inhibiting the enzyme responsible for the conversion of NH_3 to NO_3^-, therefore reducing leaching and gaseous losses of N. Studies demonstrating DCD use have identified differences in DCD effectiveness with changing temperature (Amberger 1989; Di & Cameron 2004), application rate, soil type (Singh et al. 2008), drainage (Di & Cameron 2011; Shepherd et al. 2012), rainfall (Luo et al. 2010b), organic matter content, and liming (Puttanna et al. 1999b). However, the effect of short-term soil pH alteration on DCD effectiveness has not been determined, although Puttana et al. (1999b) states that increasing the soil pH reduces DCD efficiency. Zhang et al. (2011) found that DCD was less effective at inhibiting AOB than AOA in naturally acidic soils. In an alkaline calcareous soil, Mahmood et al. (2011) found that DCD enhanced N losses from urea.

The objective of this study was to determine the effect of short term soil pH change on N_2O emissions, ammonia oxidising communities, and DCD efficiency. Previous studies identifying the effects of soil pH have been conducted in the laboratory (Thomsen et al. 1994; Clough et al. 2004; Bakken et al. 2012) or on long term/natural soil pH plots (Weslien et al. 2009; Gubry-Rangin et al. 2010; Mahmood et al. 2011; Zhang et al. 2011). Few studies have determined the effect of short term pH change on N_2O emissions, ammonia oxidising

communities, and DCD effectiveness in the field. It was hypothesised that: 1) N_2O emissions would be highest in soil with an acidic pH due to inhibition of the N_2O reductase enzyme; 2) AOB would dominate in a basic pH soil whereas; in accordance with Zhang et al. (2011), AOA would dominate in an acidic pH soil; and 3) DCD would be most effective at reducing N_2O emissions in soil with an acidic pH (Puttanna et al. 1999b).

5.2 Methods

A field trial was set up to determine the effect of soil pH on N_2O emissions, AOA and AOB communities and DCD effectiveness.

5.2.1 Field trial set up

The trial was established at Lincoln University on the 3rd of May 2012. The soil at the site was a Temuka clay loam and had been under pasture for 8 years with regular fertiliser and irrigation additions. In December 2011, prior to field trial establishment, a basal application of urea-N (50 kg/ha) and super phosphate fertiliser (500 kg/ha) was applied.

Figure 5.1 Location of field trial at Lincoln University, Christchurch (43˚38'55"S, 172˚ 28'4"E). The red rectange indicates the field plot area.

The experimental site contained 48 treatment plots (4 replicates of 12 treatments) each measuring 1.5 x 1.3 m in size (Figure 5.2). A randomised block design was used in the study and treatments were allocated to plots using a random number generator (Figure 5.3). The pH alteration occurred over the whole plot prior to installation of gas sampling and soil sampling rings.

Figure 5.2 The study site set up for pH treatment application (left) and for Urine/DCD treatment application (right).

5.2.1.1 Soil pH alteration

For soil pH alteration CaO or NaOH and HCl was used for the basic pH and acidic pH treatment respectively. The CaO, HCl and water/control treatment applications are referred to as "basic", "acidic", and "native" respectively.

Initially, the pH treatments were applied in three batches on the 4th, 7th and 9th of May 2012. For the basic pH treatment, 29.25 g of CaO was applied as a fine powder to the surface of the plots and watered in with three 10 L aliquots using a watering can. For the acidic pH treatment, 10 L of 3.6% HCl was applied three times to each acidic plot. The same volume of water was added to the control plots. The addition of treatments in three aliquots allowed the treatment to 'soak in' and prevented overland flow into neighbouring plots. Monitoring of the plots occurred for one month until the pH reached satisfactory values, pH < 5 (acidic), and pH > 6.5 (basic). However, due to heavy snowfall/rainfall in early June 2012 the treatments leached out of the plots and re-application was required.

Re-application of pH treatments occurred on the 21st of June 2012. A stronger solution of HCl was applied to the acidic plots, and 1.2 M NaOH added to the basic plots. On the 28th of

June 2012 an application of 1.2 M NaOH was repeated on the basic plots to overcome the buffering capacity of the soil and reach the required basic pH. Unsatisfactory pH changes were experienced with the use of CaO_2 due to its low solubility; hence NaOH was used in its place. However, in September the pH values began to return to the native pH, hence another reapplication of pH treatments occurred on the 18th September 2012.

Figure 5.3 Field trial study design, plots were 1.5 m x 1.3 m, including a 25 cm buffer non-sampling area (white).

5.2.1.2 Sampling equipment installation

Following soil pH alteration, stainless steel and aluminium rings 500 mm in diameter by 150 mm deep were installed in each of the 48 plots to facilitate N_2O sampling. The rings allowed a modified closed chamber method (Figure 5.4) (Hutchinson & Mosier 1981) to be employed, whereby a styrofoam and steel cap was placed inside a trough filled with water on top of the gas sampling rings. The water provided an airtight seal. The rings were left permanently in the field for the duration of the trial, while the caps were used only when N_2O samples were being taken. Complimentary rings were installed beside each of the gas sampling rings to allow soil samples to be taken for pH measurement, mineral N, DCD concentration and microbial assays.

Figure 5.4 Gas rings with water filled trough (left) and gas chamber with rubber septum and white pressure cap (right). The gas ring was left permanently in the field while the gas chamber was only used while sampling.

5.2.1.3 Urine and DCD treatment application

On the 9th August 2012, urine and DCD treatments were applied to the area within the appropriate gas sampling and soil sampling rings. The following treatments were used; control (no urine or DCD); urine (700 kg urine-N/ha); DCD (10 kg DCD/ha) and; urine plus DCD (700 kg urine-N/ha + 10 kg DCD/ha). These treatments are referred to as "control", "urine-only", "DCD-only" and "urine+DCD" respectively. Synthetic urine was used due to the large volume required (see Table 5.1 for composition). Herbage was cut by hand fortnightly or when required for ease of soil and gas sampling. The soil pH was monitoring weekly to ensure the pH was maintained at the correct level. On the 18th of September 2012 an additional application of HCl and NaOH was applied to the installed gas and soil sampling rings because the acidic and basic pH treatments had started to return to the natural soil pH.

Table 5.1 Synthetic urine composition based on Fraser et al. (1994).

Compound	Rate (g/L)
Urea	14
Glycine	3.5
Potassium bicarbonate	16
Potassium chloride	3
Potassium sulphate	2

5.2.2 Soil sampling and analysis

For each soil sampling date two soil cores (52 cm^3) were taken at a depth of 7.5 cm from each soil sampling ring. These were homogenised and subsamples were taken from each replicate to determine concentrations of DCD, NH_4^+ and NO_3^- concentrations, soil moisture and soil pH.

DCD was extracted from 5 g of soil using 25 mL of deionised water. Samples were shaken for 1 hour and centrifuged for 20 mins and then filtered using Whatman No. 41 filter paper. DCD concentration was analysed on a Shimadzu series High Performance Liquid Chromatography (Tokyo, Japan) using a cation-H guard column (Phenomenex, USA) and a 0.025 M sulphuric acid mobile phase at a flow rate of 0.6 ml/min by UV detection at a wavelength of 210 nm.

Extraction of NH_4^+ and NO_3^- was carried out through the addition of 25 mL of 2 M KCl to 5 g of soil. Samples were shaken for 1 hour and centrifuged for 10 mins and the supernatant filtered using Whatman No. 41 filter paper. NH_4^+ and NO_3^- concentrations were analysed using a flow injector analyser (FIA) (FOSS FIAstar 5000 triple channel analyser) with SoFIA software version 1.30 (Foss Tecator AB, Sweden).

Soil moisture was determined for each of the samples for each sampling date by weighing a subsample of soil (approximately 10 g), drying it at 105˚C for 24 hours and then reweighing it. Soil moisture was calculated using the following formula: ((wet soil (g) - dry soil (g))/dry soil (g)) x 100.

Soil pH was measured throughout the trial to ensure the pH was maintained <5 for the acidic treatment and >6.5 for the basic treatment. A 10 g subsample of field moist soil was taken and 25 mL of deionised water added (Blackmore et al. 1987). Samples were shaken briefly

and left overnight before the pH was read using a Mettler Toledo Seven Easy pH meter (Mettler Toledo, Switzerland).

5.2.3 Nitrous oxide sampling

Gas samples were taken for N_2O flux calculation twice per week for the duration of the trial. Samples were collected early-afternoon and samples were taken at time zero, 20 mins and 40 mins using a syringe through a rubber septum, and stored in 6 mL exetainers awaiting analysis. N_2O concentrations were analysed on a gas chromatograph (SRO8610 linked to a Filson 222XL autosampler) using an Electron Capture Detector (ECD) (SRI Instruments, USA) and quantified using stored ambient air samples. Elevated concentrations were achieved using ethylene and acetylene in stored air samples.

5.2.4 AOB and AOA assays

Soil subsamples were taken from the soil sampling rings at days 1, 7, 14, 33, 56 and 102 after the application of treatments to determine ammonia mono-oxygenase gene (*amoA*) copy numbers for AOB and AOA. Soil samples were stored at -80˚C prior to extraction.

DNA was extracted from frozen soil (0.25 g) using NucleoSpin® Soil Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. DNA was eluted with 100 µL of Buffer SE (Macherey-Nagel, Düren, Germany. LOT. PAF00456026) and stored at -20˚C before being analysed.

PCRs were set up using the CAS1200 Robotic Liquid Handling System (Corbett Life Science, Australia), and real-time PCR was performed on a Rotor-Gene™ 6000 (Corbett Life Science). 10-fold dilutions were used for the PCR. Bacterial and archaeal *amoA* genes were quantified using the primers amoA1F/amoA2R (Rotthauwe et al. 1997) and Arch-amoAF/Arch-amoAR (Francis et al. 2005) respectively, with SYBR® Premix Ex Taq™ (TaKaRa, Japan) using the thermal profiles as described in Di et al. (2009). The 20 µL reaction mixture contained 10 µL of SYBR® Premix Ex Taq™ including primers, and 1.5 µL of template DNA. To confirm PCR product specificity, a melting curve analysis was carried out, by measuring fluorescence continuously as the temperature increased from 50 to 99˚C. Data analysis was carried out using Rotor-Gene™ 6000 series software 1.7.

Standard curves for real-time PCR assays were developed using the following method. Briefly, the bacterial and archaeal *amoA* genes were PCR amplified from the extracted DNA with the aforementioned primers. The PCR products were purified using the PCR clean-up kit (Axygen) and cloned into the pGEM-T Easy Vector (Promega, Madison, WI) and the resulting ligation mix transformed into *Escherichia coli* JM109 competent cells (Promega) following the manufacturer's instructions. Plasmids which were used as standards for quantitative analyses were extracted from the correct insert cloners from each target gene and sent for sequencing. A Qubit™ fluorometer (Invitrogen NZ) was used to determine the plasmid DNA concentration and the copy numbers of target genes calculated. Tenfold serial dilutions of the known copy number of the plasmid DNA were then subjected to a real-time PCR assay in triplicate to generate an external standard curve and to ensure amplification efficiency.

5.2.5 Climate data

Daily rainfall and daily maximum temperature for the trial period, and long term climate data, was collected from the Broadfields, Lincoln climate station using the National Climate Database (www.cliflo.niwa.co.nz). Soil temperature data was collected from the data logger at the lysimeter paddock, Lincoln University.

5.2.6 Statistical analysis

Mean values and standard errors of the means for N_2O emissions, NO_3^- concentrations and DCD concentrations were calculated based on the four replicates for each treatment using Microsoft Excel 2010 (Microsoft Corporation, USA). Least significant differences and p-values were calculated using general analysis of variance in Genstat© (Version 15.1, VSN International Ltd, U.K.). N_2O values were log-transformed.

5.3 Results

5.3.1 Climate

During the trial period, there were two days where the rainfall exceeded 20 mm (Figure 5.5). The highest rainfall recorded was 50.6 mm on 12th of August (day 1 of field trial). During the trial period there were a total of 67 days where no rainfall was recorded. Long term total rainfall for September was lower than what was recorded during the trial (Table 5.2). In contrast, long term rainfall during August and October was higher. Soil water content followed the rainfall data with peaks in soil moisture seen after the rainfall occurred. The highest soil water content was 46% on the 23rd of August (day 14). The general trend showed a slow decrease in soil water content throughout the trial period (Figure 5.5A).

The average maximum air temperature throughout the trial was 14.7^{0}C (Figure 5.5B). The highest average daily maximum air temperature recorded was 23.8^{0}C on the 1st of November and the lowest was 8.3^{0}C on the 13th of August. Average monthly maximum air temperatures for the trial period were similar to the long term averages with the largest difference being 0.5^{0}C (Table 5.2).

Fluctuations in soil temperature followed the fluctuations seen in the average maximum air temperature (Figure 5.5B). The average soil temperature for the trial period was 10.9^{0}C. The lowest soil temperature was 6.8^{0}C on the 13th of September. The highest soil temperature was 17.3^{0}C on the 2nd of November.

Table 5.2 Total rainfall and mean daily max air temperature long term data (1981-2010) and trial data for the full months of the trial period at the Lincoln Broadfield climate station.

		August	September	October
Total Rainfall	Long term	61.0	39.7	50.6
	Trial	107	29	70
Mean Daily Maximum Air Temperature	Long term	12.3	14.7	16.6
	Trial	12.5	15.2	16.5

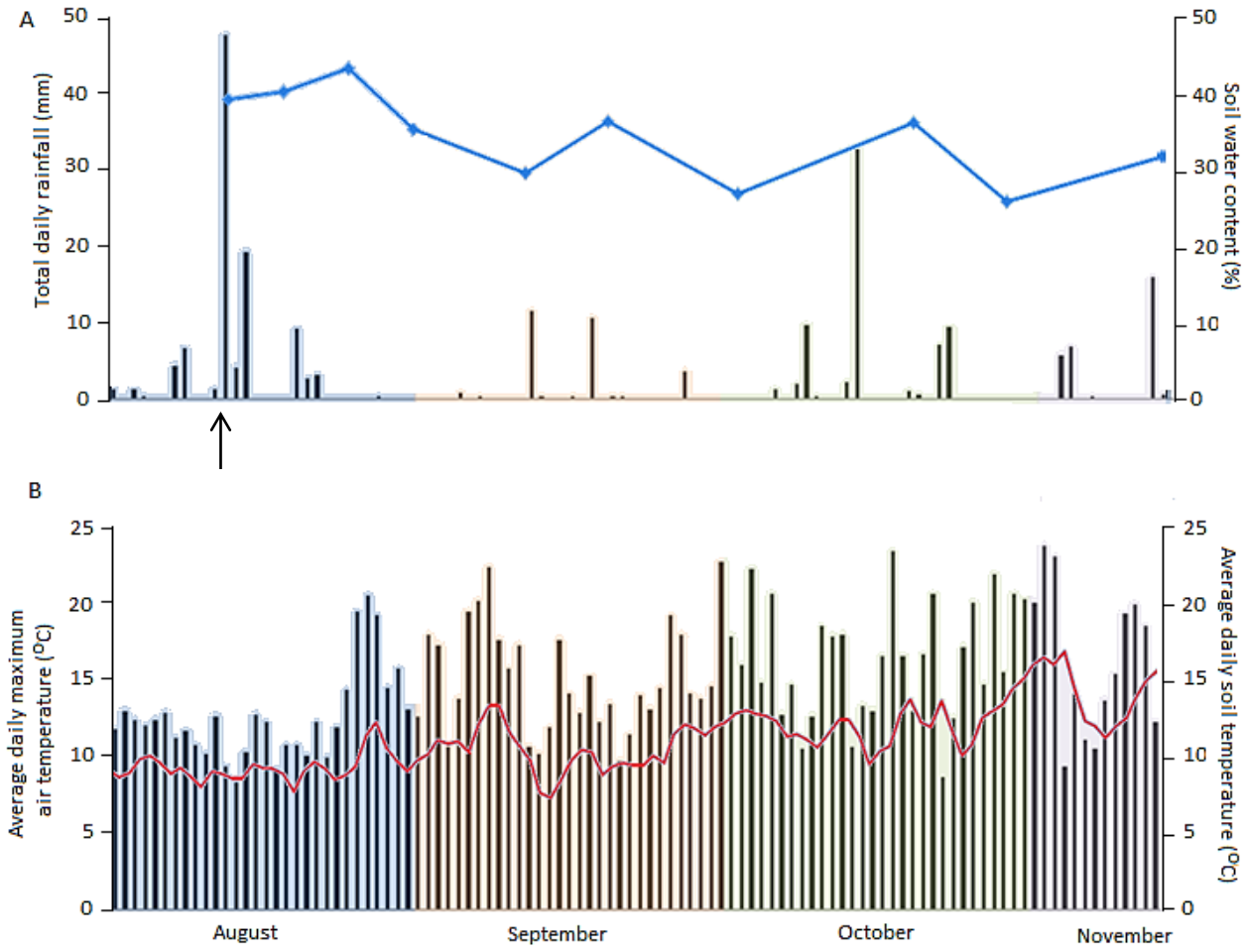

Figure 5.5 (A): Total daily rainfall (bars) and measured soil water content (%) (blue line); (B): mean daily max air temperature (bars) and mean daily soil temperature at 10 cm depth (red line) for the field trial period. Arrow indicates start of trial (9/08/2012).

5.3.2 Soil pH

The soil pH was significantly different between pH treatments (Figure 5.6, Appendix 3). The application of urine did not significantly alter the pH of the basic or native pH soil. However 7 days following urine application, the soil pH did increase in all pH treatments, with a significant ($p < 0.05$) increase of 1.2 units seen in the acidic soil (Figure 5.6). DCD did not significantly alter the soil pH throughout the trial.

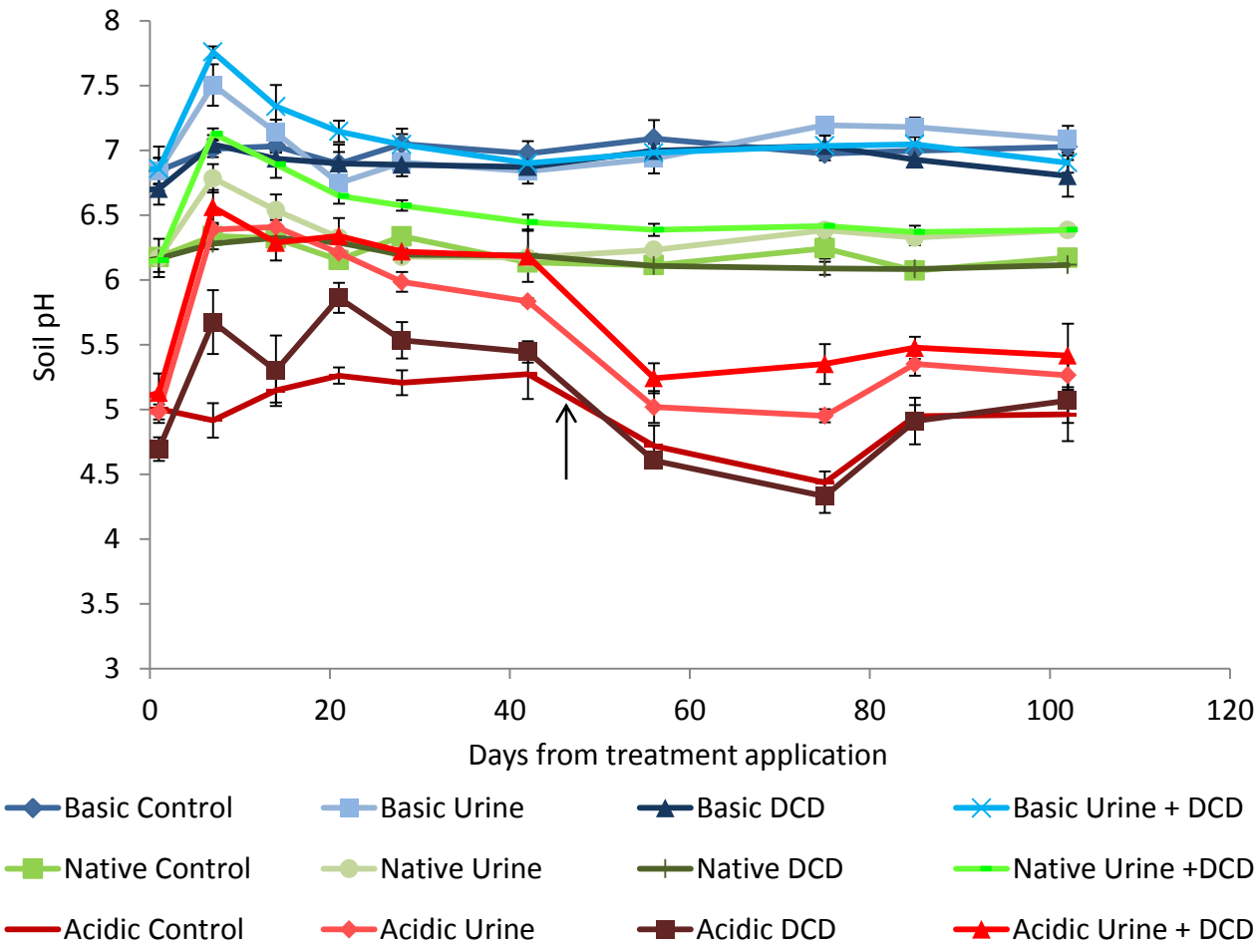

Figure 5.6 Soil pH after urine and DCD treatment application (day 0). Arrow indicates reapplication of pH treatments (18th September 2012) after the application of urine and DCD treatments. Error bars show standard error of the mean (SEM).

5.3.3 N_2O emissions

5.3.3.1 Daily N_2O emissions

Soil pH did not significantly affect the flux rate of N_2O from a Temuka clay loam over the field trial period (Figure 5.7). The rate of N_2O emissions from the control and DCD-only treatments remained relatively constant throughout the monitoring period, and there was no significant difference between pH treatments (Figure 5.7A & B).

The highest emissions were recorded in the urine-only treatments, where emissions peaked at day 7 from the basic pH soil with 0.26 mg N_2O-N/m^2/hr., and from the acidic pH with 0.64 N_2O-N/m^2/hr. (Figure 5.7C). For the native pH, peak emissions of 0.34 N_2O-N/m^2/hr. occurred on day 13.

Peak emissions from the urine+DCD treatment occurred on day 13 for all soil pH treatments (Figure 5.7D). When compared to the urine-only treatment peaks at day 7 and day 13, the urine+DCD peaks were 54% lower in the basic pH soil, 50% lower in the native pH soil and 71% lower in the acidic soil. However, only the native pH soil had a significant reduction in peak N_2O emissions with DCD application ($P = 0.08$), due to high sample variance in the acidic and basic pH treatments.

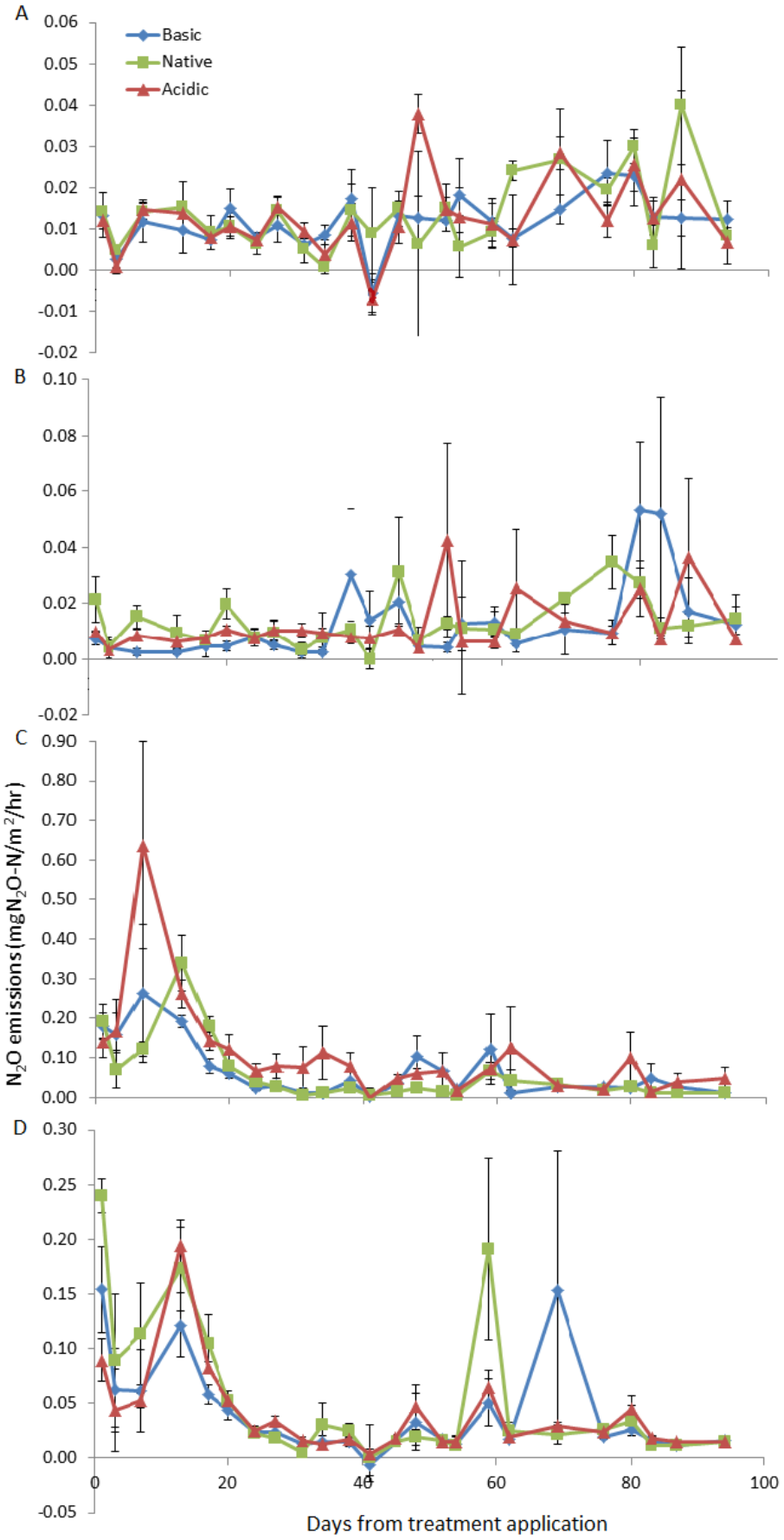

Figure 5.7 N_2O flux for basic, native and acidic pH treatments; (A): control; (B): DCD-only; (C): Urine-only; and (D): urine+DCD during field trial. Error bars show SEM.

5.3.3.2 Total N_2O emissions

The control plots, on average, emitted a total of 300 g N_2O-N/ha over the study period. With the addition of urine, total N_2O emissions significantly increased, with the total N_2O emitted being significantly higher in the acidic pH soil compared to the native ($p = 0.05$) and basic pH soil ($p = 0.01$) (Figure 5.8). The basic pH soil emitted a total of 1445 g N_2O-N/ha; the native pH soil emitted a total of 1291 g N_2O-N/ha, whereas the soil with an acidic pH emitted almost twice as much N_2O at 2450 g N_2O-N/ha. In the native and basic pH treatment, the addition of DCD did not significantly reduce the total amount of N_2O emitted compared to the urine-only treatment (Figure 5.8). However in the acidic pH soil, addition of DCD reduced total N_2O emissions by 64%, from 2450 in the urine-only treatment to 878 g N_2O-N/ha in the urine+DCD treatment. The addition of DCD in the absence of urine did not significantly affect the total amount of N_2O emitted, when compared to the control.

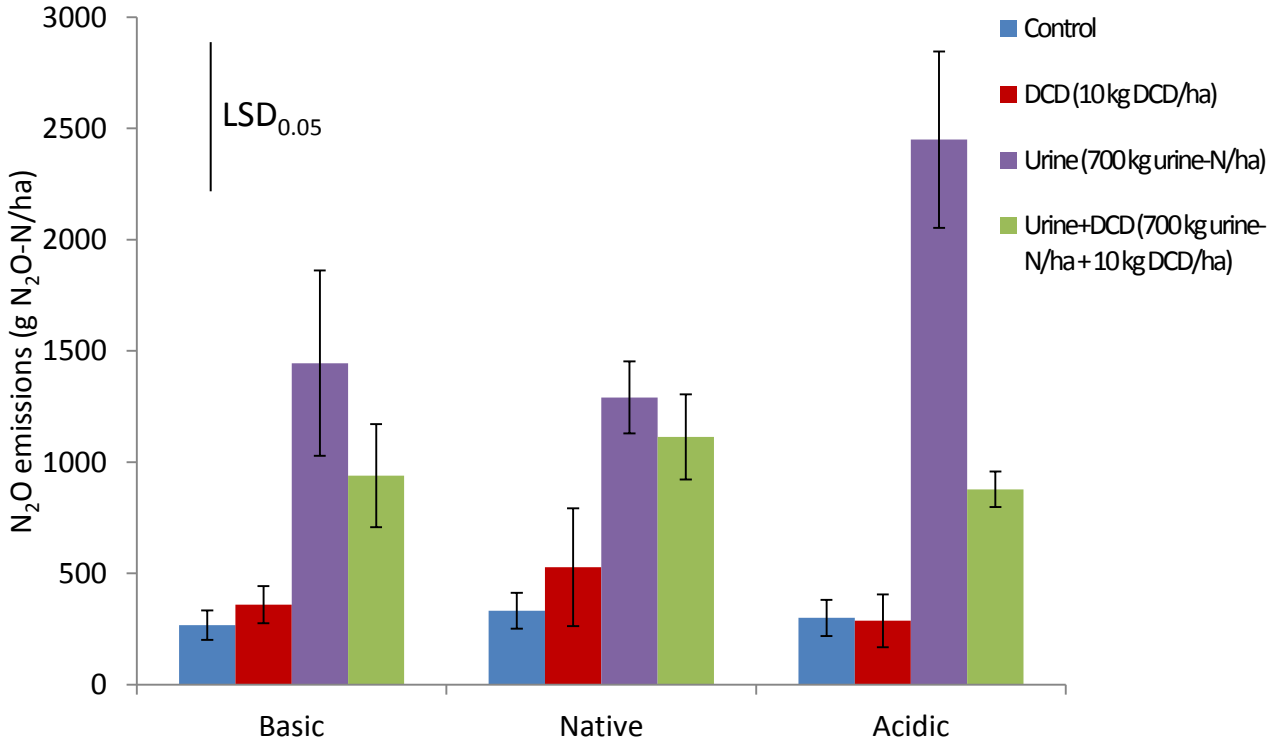

Figure 5.8 Total N_2O emitted during the trial period for basic, native and acidic pH treatments. Standard errors show SEM. $LSD_{0.05}$ demonstrates the least significant difference with 95% certainty.

5.3.4 Soil NO_3^- concentration

In the control, DCD-only and urine+DCD treatments, NO_3^- concentrations for the native and basic pH soil followed the same temporal trend (Figure 5.9). In contrast, the NO_3^- concentrations in the acidic pH soil remained stable throughout the trial period.

In the control treatment, soil pH did not significantly affect NO_3^- concentrations (Figure 5.9A). However, with urine addition, the NO_3^- concentrations increased significantly ($p < 0.05$) in the basic and native pH soil (Figure 5.9C, Appendix 3). The highest NO_3^- concentration was seen in the basic pH soil at day 14 with a peak of 84.1 mg NO_3^--N/kg of dry soil which was significantly ($p < 0.05$) higher than the acidic and native pH soils (Appendix 3).

In the urine+DCD treatment the native and basic pH soil followed similar trends and had higher NO_3^- concentrations than the acidic pH soil from day 14 to day 56 (Figure 5.9D). When comparing the urine-only and urine+DCD treatments, the NO_3^- concentrations were significantly ($p < 0.05$) lower at day 14 in the basic pH soil only, with NO_3^- concentrations decreasing from 84.1 mg NO_3^--N /kg of dry soil in the urine-only treatment to 19.5 mg NO_3^--N /kg of dry soil in the urine+DCD treatment (Figure 5.9C&D, Appendix 3). In contrast, there was no significant difference in NO_3^- concentration at day 14 in the native and acidic pH soil with DCD addition (Appendix 3). However when comparing the average reduction in NO_3^- concentrations over the trial period, the acidic pH had the highest average reduction of 65% with the native and basic pH having average reductions of 28% and 42% respectively.

The addition of DCD in the absence of urine did not significantly affect soil NO_3^- concentrations compared to the control at any soil pH (Figure 5.9A&B).

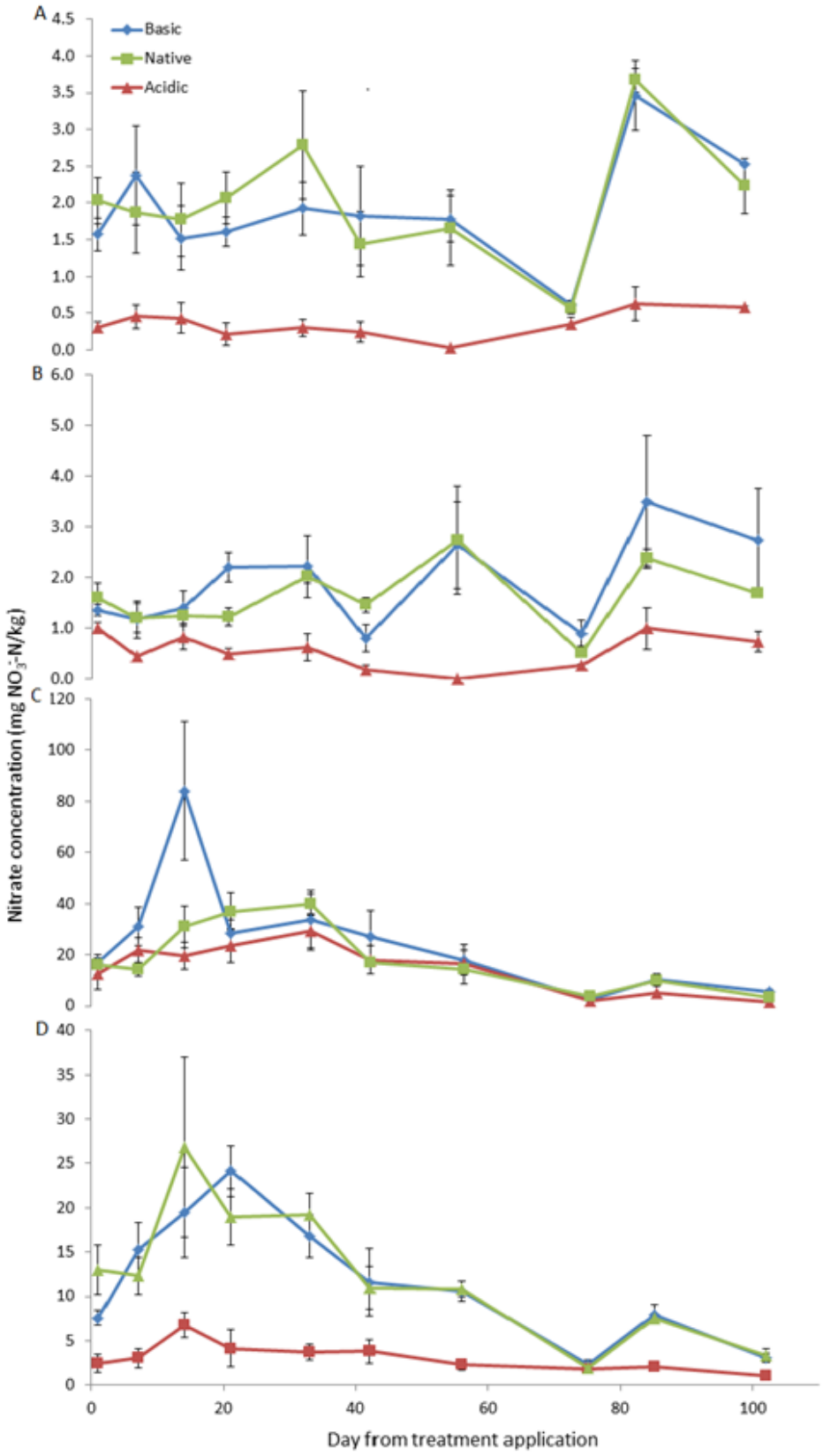

Figure 5.9 Nitrate concentrations for (A): control; (B): DCD-only; (C): urine-only; (D): urine+DCD. Error bars indicate SEM.

5.3.5 Soil NH_4^+ concentrations

Overall, soil pH had a main effect on NH_4^+ concentrations (linear trend p-value = 0.003) (Appendix 3). In the control and DCD-only treatment, NH_4^+ concentrations remained at similar values for all the pH soils (Figure 5.10A & B). However, with the addition of urine, the NH_4^+ concentrations increased compared to the control and DCD-only treatments with significant ($p < 0.05$) increases in the acidic pH soil (Figure 5.10C, Appendix 3).

Similar to the NO_3^-concentrations, the native and basic pH soils followed similar trends for NH_4^+ concentrations in the urine+DCD and urine-only treatment. However, in the acidic pH soil, NH_4^+ concentrations were higher than in the native and basic pH soil. In the urine+DCD treatment, the native and basic pH soil had initially higher NH_4^+ concentrations compared to the urine only treatment. Furthermore, the NH_4^+ concentration stayed higher for longer with the addition of DCD in all pH treatments.

The application of DCD without urine had no significant effect on NH_4^+ concentrations compared to the control (Figure 5.10B).

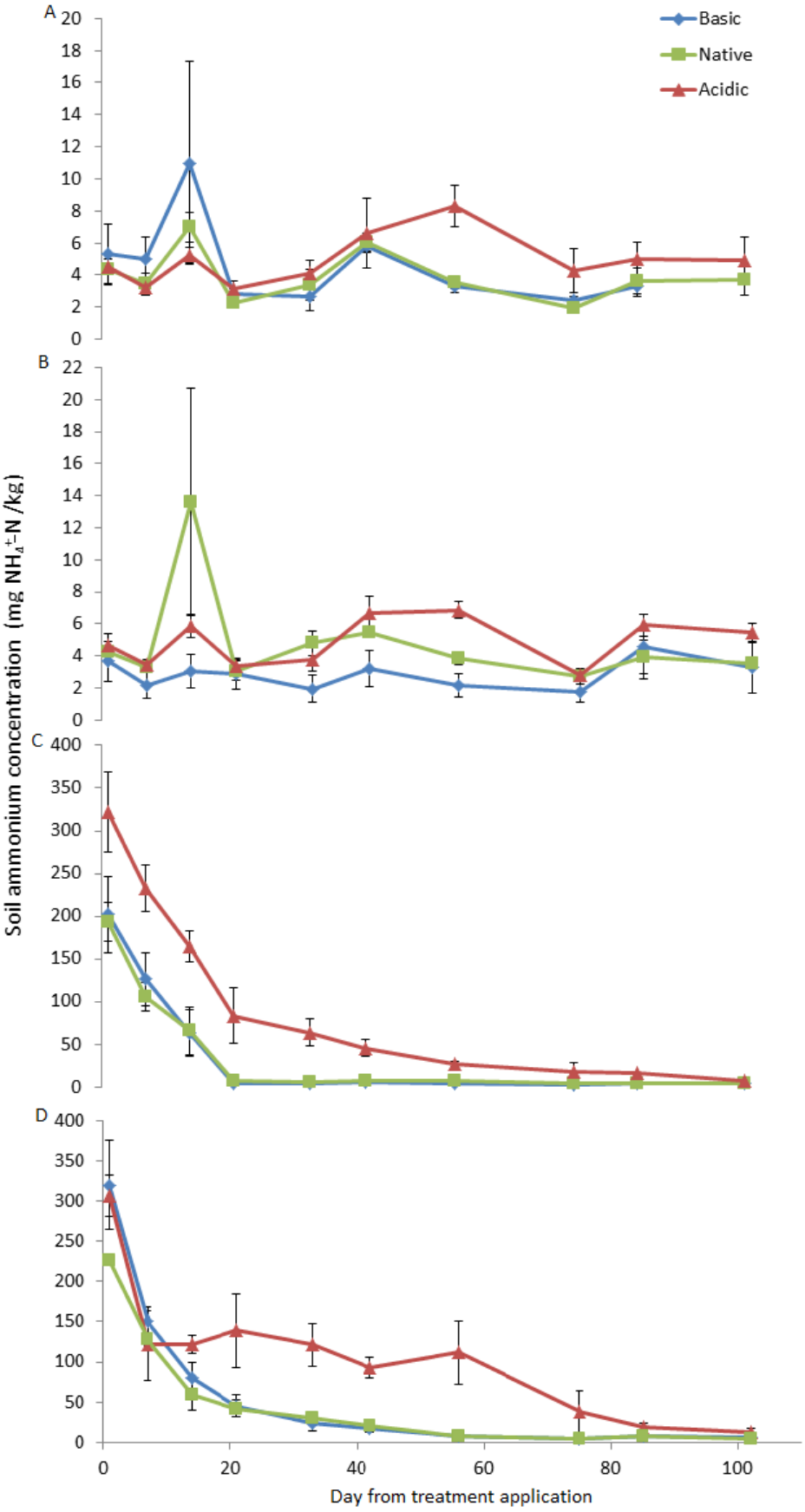

Figure 5.10 Soil ammonium concentration for basic, native and acidic soil pH; (A): control; (B): DCD-only; (C): urine-only; and (D): urine+DCD. Error bars indicate SEM.

5.3.6 DCD concentration

Average DCD concentration for all treatments amended with DCD, degraded at an exponential rate, with a half-life of 14 days (using average concentration of DCD at day 1 for all treatments). For the urine+DCD treatment, DCD became undetectable in the basic and native pH soil, at day 85, while DCD could be detected in the acidic pH soil throughout the whole trial period (Figure 5.11). For the DCD-only treatments, DCD became undetectable in the native and basic soil pH soil at day 75.

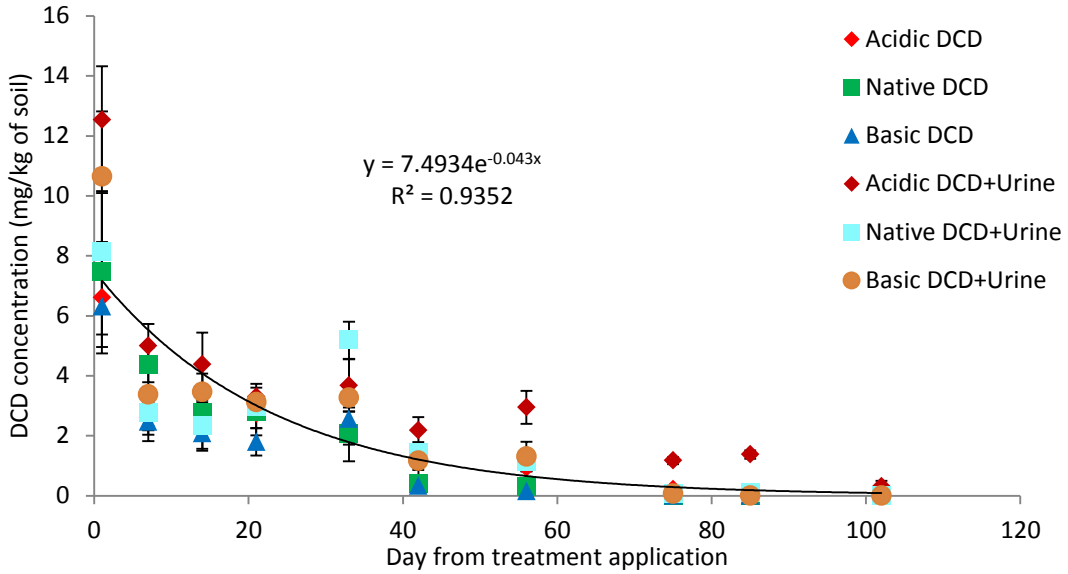

Figure 5.11 DCD concentration in DCD-only and DCD+urine treatments. Trend line shows exponential trend for averaged data across all DCD-only and DCD+urine treatments at each sampling date. Error bars indicate SEM.

5.3.7 Ammonia oxidising community abundance

5.3.7.1 Ammonia oxidising bacteria

In the control treatment, pH had no significant effect on AOB *amoA* gene abundance (Figure 5.12). Peak AOB abundances were seen at day 14 and day 90 in the basic pH soil, and day 7 in the acidic and native pH soil (Figure 5.13C). In the urine-only treatment, from day 45 until the end of the trial period, AOB abundances continued to increase in the basic pH, whereas in the native and acidic pH the AOB abundances remained stable (Figure 5.13C).

In the basic and native pH soil the addition of urine significantly ($p < 0.05$) increased the AOB *amoA* gene abundance compared to the control (Figure 5.12). The acidic pH had no significant increase. Throughout the trial, in the urine-only treatment, the basic pH soil had significantly ($p < 0.05$) higher AOB abundance compared to the acidic pH soil (Figure 5.13C).

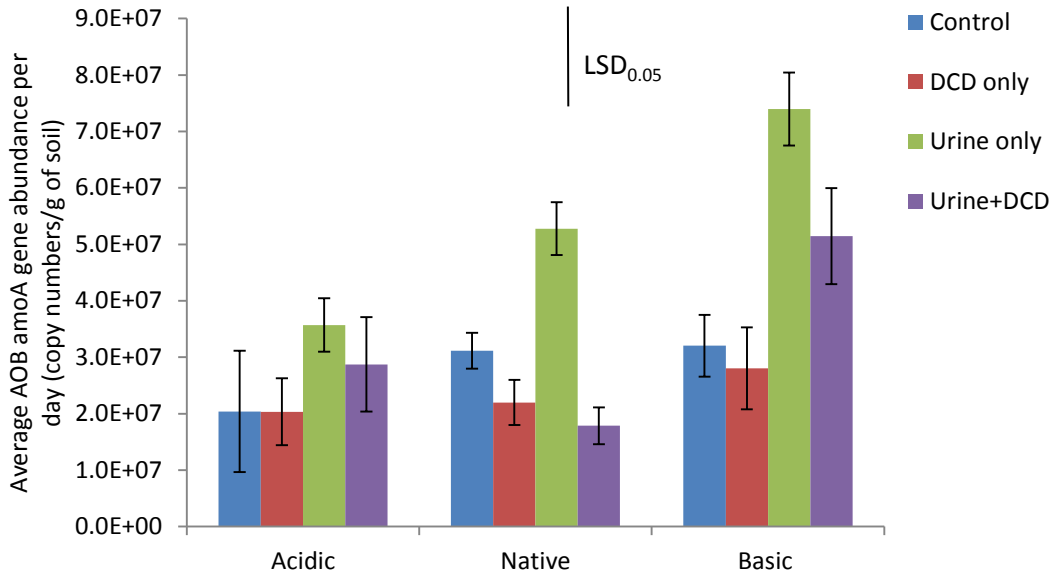

Figure 5.12 Weighted average AOB amoA gene abundance (*amoA* copy numbers/g of soil/ day). Error bars indicate SEM and LSD indicates least signficant difference (p = 0.05).

In the urine-only and urine+DCD treatments, a significant ($p < 0.05$) reduction in AOB abundance was seen in the basic pH soil and acidic pH soil (Figure 5.12). In the native pH soil there was no significant reduction in AOB *amoA* gene abundance with DCD addition.

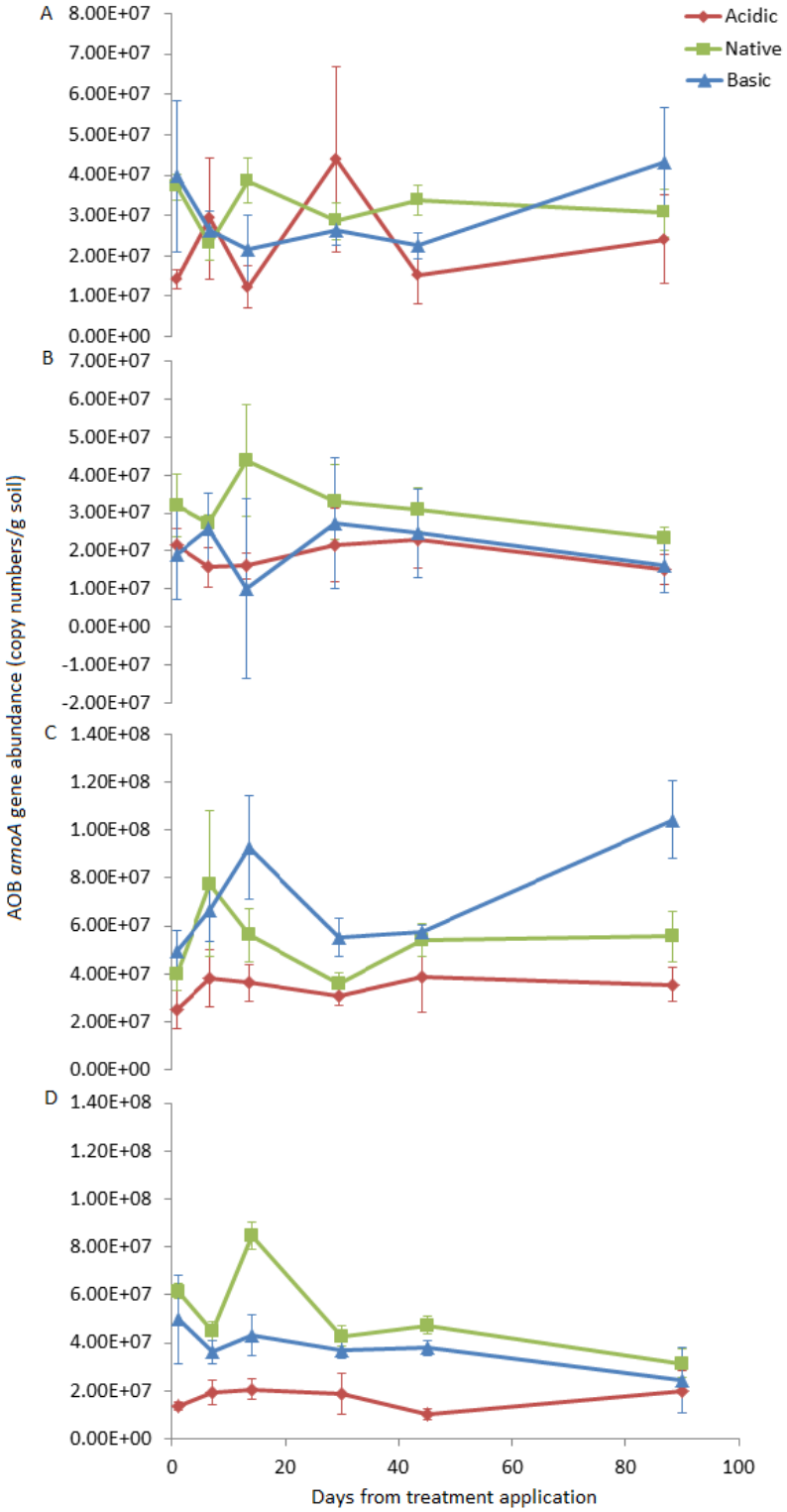

Figure 5.13 AOB amoA gene abundance for basic, native and acidic pH treatments (A): control; (B): DCD-only; (C): urine-only; and (D): urine+DCD. Error bars indicate SEM.

5.3.7.2 Ammonia oxidising archaea

In the control treatment, pH had no significant effect on AOA *amoA* gene abundance (Figure 5.14, 5.15). However with the addition of urine, AOA *amoA* gene abundances were significantly ($p < 0.05$) higher in the acidic pH soil compared to the basic pH soil (Figure 5.4). In the urine-only treatment, the acidic pH soil AOA *amoA* gene abundance decreased from day 1 until day 28, and then increased from day 28 until day 90 (Figure 5.15C). In contrast, in the native and basic pH soil AOA abundance remained the same from day 28 until the end of the trial (Figure 5.15C). At day 90, AOA *amoA* gene abundance was significantly ($p < 0.05$) higher in the acidic pH soil with $3.59x10^7$ copy numbers/g dry soil, compared to the basic pH soil which had an abundance of $1.91x10^7$ copy numbers/g dry soil (Figure 5.15C, Appendix 3).

In the urine+DCD treatment, AOA *amoA* gene abundance was significantly lower compared to the urine-only treatment in the acidic pH soil (Figure 5.14). In contrast, in the basic and native soil pH soil there was no significant change in AOA *amoA* gene abundance with DCD addition.

The application of DCD alone had no effect on AOA *amoA* gene abundance in all soil pH's compared to the control (Figure 5.14).

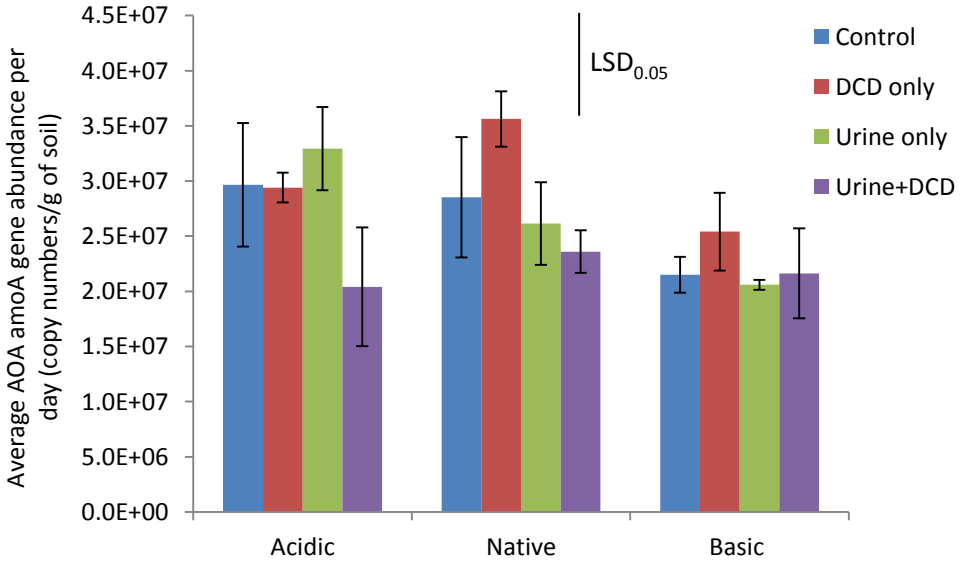

Figure 5.14 AOA amoA gene abundance for (A): acidic pH; (B): native pH; (C): basic pH. (E). Error bars indicate SEM.

AOA *amoA* gene abundance was lower than the AOB *amoA* gene abundance in all the basic pH treatments. However in the acidic pH AOA *amoA* gene abundance was higher than AOB *amoA* gene abundance, with the exception of the urine-only and urine+DCD treatments

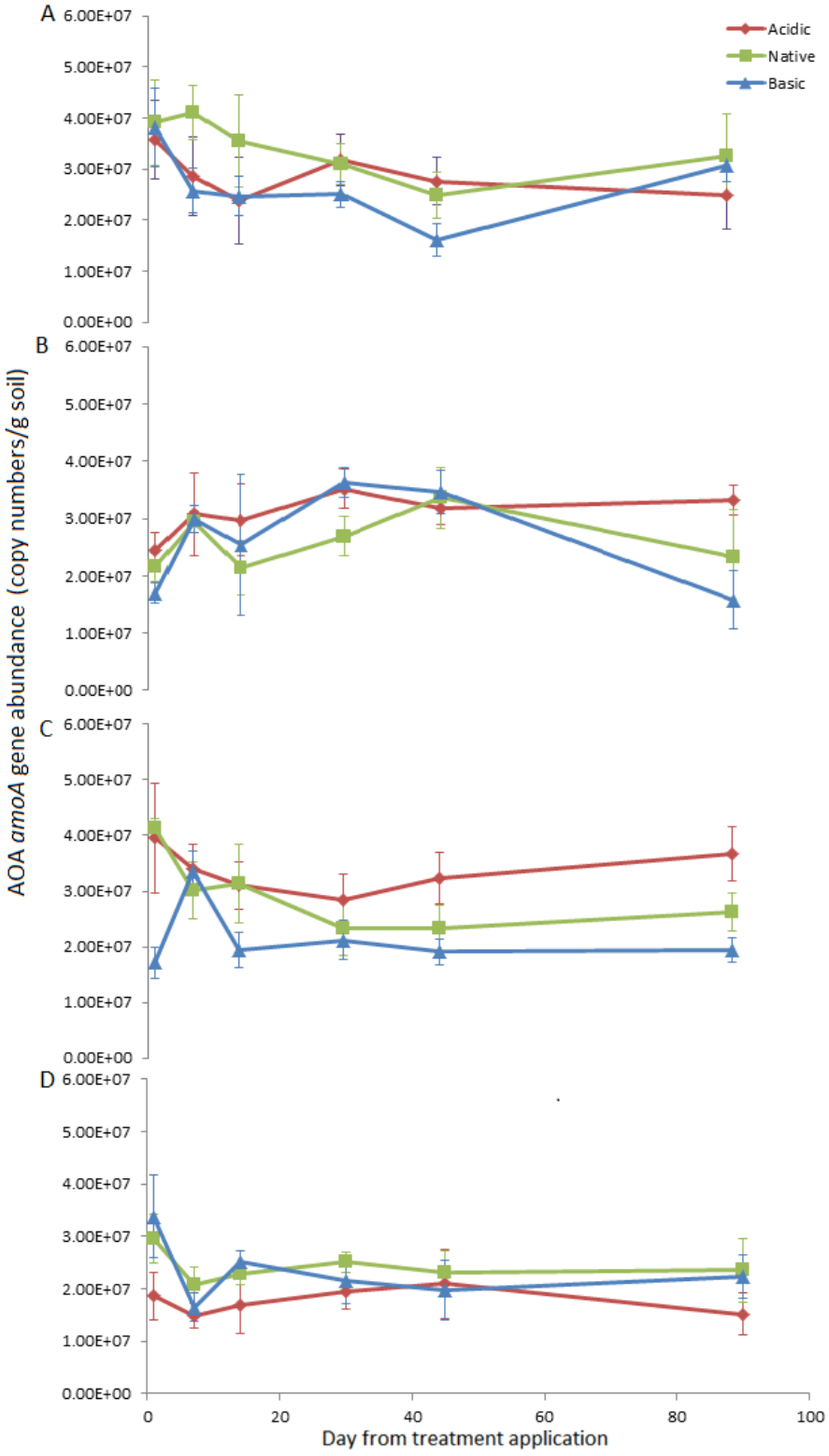

Figure 5.15 AOA *amoA* gene abundance for control (A): control; (B): DCD-only; (C): urine-only; and (D): urine+DCD. Error bars indicate SEM.

5.4 Discussion

5.4.1 N_2O emissions and soil pH

Total N_2O emissions with urine addition were significantly ($p < 0.05$) higher in the acidic pH soil compared to the native and basic pH soil. This is similar to the findings of Weislien et al. (2009) who found a strong negative correlation between N_2O emissions and increasing soil pH. The pH of a soil affects N_2O production by interrupting denitrification pathways (Wijler & Delwiche 1954). Denitrification is the complete conversion of NO_3^- to N_2, through the intermediates nitrite (NO_2^-), nitric oxide (NO) and N_2O (Wrage et al. 2001). At a low pH, the N_2O reductase enzyme, responsible for the conversion of N_2O to N_2, can become inhibited, which causes incomplete denitrification and a higher production of N_2O (Knowles 1981). Furthermore, in acidic soils ammonia oxidisers can yield more N_2O (Jiang & Bakken 1999; Morkved et al. 2007), increasing the nitrification contribution to N_2O emissions in the acidic pH soil. Thus, in the acidic pH soil a higher proportion of N_2O would have been produced, whereas in the basic pH soil a higher proportion of N_2 would have been produced, decreasing the basic pH soil N_2O emissions. This is in agreement with Stevens et al. (1998) who found that as the soil pH increased, a greater proportion of N_2 was produced. Similarly, Clough et al. (2004) found cumulative N_2 fluxes increased with increasing soil pH in a saturated soil. During the N_2O peak, the soil water content was at its highest for the trial period. Increased soil moisture during the first week of the field trial, could have caused higher N_2O peaks in the acidic pH at day 7, and higher N_2 emissions from the basic pH soil. This agrees with the results of Clough et al. (2004), where highest N_2O emissions were seen in an acidic soil when the soil was at field capacity.

5.4.2 Soil NO_3^-, NH_4^+ concentrations and ammonia oxidising communities

With the addition of urine, soil NH_4^+ concentrations were significantly ($p < 0.05$) higher in the acidic pH soil compared to the basic and native pH soils. In contrast soil NO_3^- concentrations were significantly higher in the basic pH soil compared to the acidic and native pH soils. This is in agreement with Dancer et al. (1973) who found that acidic soils accumulated more NH_4^+ and basic soils accumulated more NO_3^-. They believed this was caused by a reduction in nitrifier abundance as well as slower nitrification rates in acidic soils. In this study, with the addition of urine, AOB *amoA* gene abundance was significantly ($p < 0.05$) lower in the acidic pH soil compared to the basic pH soil. This is believed to be caused by a reduction in NH_3 availability through ionisation to NH_4^+, at a low pH (Frijlink et al. 1992). Nicol et al. (2008)

found that AOB *amoA* gene abundance increased with increasing pH due to enhanced NH_3 concentration.

In agreement with Nicol et al. (2008), Gubry-Rangin et al. (2010) and Zhang et al. (2011) AOA *amoA* gene abundance in this study was significantly ($p < 0.05$) higher in the acidic pH soil compared to the basic pH soil. Initially, in the acidic soil with urine addition, AOA abundance decreased, suggests that the AOA were initially inhibited by the addition of urine as described by Di et al. (2010b). However, as the urine was hydrolysed, and the NH_4^+ and NO_3^- concentrations reduced, the AOA abundance became (at day 90) compared with the basic and native pH soil. Furthermore, in the acidic pH soils, AOA abundance was higher than AOB abundance, except for those treatments with urine addition. This implies that AOA can become dominant with long or short term decreases in soil pH and supports the hypothesis that AOA prefer low pH, low nutrient environments (Di et al. 2009; Erguder et al. 2009; Di et al. 2010a).

The addition of DCD to the urine only treatment significantly ($p < 0.05$) lowered the AOB *amoA* gene abundance in the acidic and basic pH soils compared to the urine-only treatment, with the largest reduction in abundance being in the basic pH soil. The greater reduction of AOB *amoA* gene abundance seen in the basic pH soil could be caused by a higher overall abundance compared to in the native and acidic pH soil. The significant decrease in the acidic pH could be caused by an increase in DCD effectiveness a low soil pH (Puttanna et al. 1999b). Overall, DCD had no significant effect in the native pH soil; however at day 90 there was a reduction in AOB *amoA* gene abundance compared to the urine only treatment.

DCD did not have any effect on AOA *amoA* gene abundance in the native and basic pH soil. This is in agreement with Di et al. (2009) who found in a soil with a pH of 5.9, DCD decreased AOB *amoA* gene abundance, while AOA *amoA* gene abundance was unaffected. In contrast to the findings of Di et al. (2009), a decrease in AOA *amoA* gene abundance was seen with DCD addition in the acidic pH soil ($pH < 5$) of this study. This supports the findings of Zhang et al. (2011) who found in natural strongly acidic soils ($pH < 4.5$), DCD inhibited AOA *amoA* gene abundance. The inhibition of AOA in the acidic pH soil indicates at a pH less than 5 AOA will increase their contribution to nitrification and therefore be inhibited by DCD. In addition, the inhibition of AOA *amoA* gene abundance in the acidic soil supports the hypothesis that DCD may be a more effective nitrification inhibitor at a lower soil pH (Puttanna et al. 1999b).

5.4.3 DCD effectiveness

The application of DCD to the urine treatments was highly effective in the acidic pH soil where total N_2O emissions were significantly ($p < 0.05$) reduced by 64% (Table 5.3) and NO_3^- concentrations were reduced by 65% when averaged over the trial period. Furthermore, NH_4^+ concentrations remained higher and for longer in the acidic pH soil compared to the basic and native pH soils. In the acidic soil, the reduction in total N_2O emissions is similar to other studies which reported reductions with DCD application of 60-90% for N_2O emissions (Di & Cameron 2002, 2006; Smith et al. 2008; de Klein et al. 2011) and 60% for NO_3^- concentrations (Di and Cameron 2002, 2005).

Total N_2O emissions were not significantly reduced in the basic pH soil. This could be caused by complete denitrification producing a higher proportion of N_2, rather than N_2O, at a higher soil pH (Stevens et al. 1998; Clough et al. 2004). In addition, to further reduce DCD effectiveness, DCD was degraded faster in the native and basic pH soils, becoming undetectable at day 84. In contrast, in the acidic pH soil, DCD was detectable for the duration of the trial (Table 5.3). Higher DCD degradation rates in the basic pH soil could have been caused by an increase in the abundance and activity of general microbial communties in the basic pH soil (Slangen & Kerkhoff 1984) causing higher microbial degradation of DCD. This agrees with Puttanna et al. (1999b) who concluded that nitrification inhibitors in limed soils would be less effective due to rapid biodegradation by greater microbial activity.

Table 5.3 Summary of DCD effectiveness at each soil pH treatment. Effectiveness was determined by comparing values for urine-only and urine+DCD treatments (+ increase, – decrease and n.s not significant (p > 0.05). DCD longevity was calculated as day where DCD became undetectable in the urine+DCD treatment (< 0.05 mg DCD/L).

Soil pH	Peak N_2O reduction	Total N_2O reduction	Average NO_3^- reduction	DCD longevity (days)
Basic	-54%	n.s	-42%	85
Native	-50%	n.s	-28%	102
Acidic	-71%	-64%	-65%	>102

The average half-life of DCD was 14 days. This half-life is low compared to DCD's half-life of 72 days at 10^0C reported in Kelliher et al. (2008) and 113 days at 8^0C reported in Di and Cameron (2004). The reduced half-life seen in this trial could have been caused by warmer temperatures and high rainfall on day 1 of the field trial. An application of DCD is

recommended during late autumn-winter-early spring when the soil temperature is cooler, causing DCD to remain in the soil for longer, and thus be more effective (Di & Cameron 2004). The average maximum air temperature in this trial was 14.7˚C. Higher air temperatures (average 16 ˚C) in the latter half of the field trial period caused the soil to warm and this may have increased the degradation of DCD. In addition, high rainfall, especially over short time periods, can have implications for the effectiveness of DCD (Luo et al. 2010b) as it can cause rapid movement of DCD out of the "active zone" via macropore flow (Shepherd et al. 2012). As the sampling depth was only 7.5 cm some of the DCD could have been leached below the sampling depth. Furthermore, as DCD degradation is a biological process, increases in soil moisture and warmer temperatures can increase microbial activity, therefore enhancing DCD biodegradation (Kelliher et al. 2008).

5.5 Conclusions

Higher total N_2O emissions in the acidic pH soil amended with urine support the hypothesis that N_2O emissions would be greater in the low pH soil. The higher N_2O emissions from the acidic pH soil are thought to be caused by inhibition of the N_2O-reductase enzyme (Weslien et al. 2009) and the soil water content at the time of peak emissions (Clough et al. 2004). In contrast, in the basic pH soil, lower N_2O emissions are thought to be caused by a higher production of N_2 due to complete denitrification (Stevens et al. 1998).

The hypothesis that AOB will increase in soils with a basic pH and AOA will increase in soils with an acidic pH is supported by the data from this study. AOB *amoA* gene abundance was significantly ($p < 0.05$) higher in the basic pH soil amended with urine compared to the native and acidic pH treatments. In contrast, AOA *amoA* gene abundance was significantly ($p < 0.05$) higher in the acidic pH soil amended with urine compared to the native and basic pH treatments. This is in agreement with Zhang et al. (2011), Gubry-Rangin et al. (2010), and Nicol et al. (2008). The initial decrease in AOA *amoA* gene abundance with urine addition supports Di et al. (2010b), however as the urine was hydrolysed AOA abundance increased further in the acidic pH soil, suggesting AOA prefer low pH, low nutrient environments (Di et al. 2009; Erguder et al. 2009; Di et al. 2010a). To further support this, AOA abundance was only higher than AOB abundance in the acidic pH soil in the absence of urine, demonstrating that in high N soils AOB will dominate nitrification (Di et al. 2009).

The addition of DCD reduced AOB *amoA* gene abundance in the acidic and basic soils as found by Di et al. (2009). However, in contrast, AOA numbers were only reduced in the acidic soil. This agrees with Zhang et al. (2011) who found a reduction in AOA numbers with DCD in a naturally strongly acidic soil.

Results from this field study found that DCD had a half-life of 14 days. This high degradation rate could be caused by a combination of the high rainfall at the start of the trial, which may have caused leaching of DCD out of the topsoil (0 - 7.5 cm) via macropore flow (Shepherd et al. 2012), and higher soil and air temperatures throughout the trial. DCD was most persistent and effective in the acidic pH soil, where total N_2O emissions and NO_3^- concentrations were significantly reduced. In contrast, in the native and basic pH soils, DCD became undetectable at day 85 and was less effective at reducing N_2O emissions and NO_3^- concentrations. This is thought to be caused by higher overall microbial activity increasing

microbial degradation of DCD in the basic and native pH soil. This finding agrees with those of Puttanna et al. (1999b) and Mahmood et al. (2011) and supports the hypothesis that DCD would be most effective at a low soil pH.

Chapter 6

General conclusions and directions for future research

6.1 General conclusions

6.1.1 N_2O Emissions

In the incubation trial (Chapter 4), aggregate size had no overall effect on total nitrous oxide (N_2O) emissions from a grazed pasture soil. However, the N_2O peaks and temporal emissions were significantly different between aggregate sizes amended with urine. Small aggregates (1-2 mm) produced a lower emission peak compared to medium (2-4mm) and large aggregates (4-5.6 mm), and continued to produce N_2O at a higher rate after day 66. Higher N_2O emission peaks in the medium and large aggregates are thought to be caused by larger anaerobic centres. Larger aggregates have lower O_2 diffusivity creating anaerobic 'hotspots' for denitrification to occur. A higher N_2O emission in the large and medium aggregates demonstrates that anaerobic centres may be present in aggregates as small as 4 mm which is in agreement with Sexstone et al. (1985). The initial peak in N_2O emissions supports the hypothesis that larger aggregates would have higher N_2O emissions, however overall the hypothesis has to be rejected as total N_2O emissions were not significantly different. The 'switch" in N_2O emissions at day 66 caused the total N_2O emissions to be the same between all aggregate sizes. The increase in emissions from the small aggregates amended with urine at day 66 is thought to be caused by aggregate instability in small aggregates. The addition of urine can cause partial dispersion of aggregates due to its high salt content and the increase in soil pH after urine addition (Uchida et al. 2008). As small aggregates have a high surface area to volume ratio, they are more susceptible to chemical disruption which can release previously unavailable carbon into solution (Gregorich et al. 1989). Thus, in agricultural soils, although large and medium aggregates will have a higher initial N_2O emission, the greater susceptibility of small aggregates to disruption with urine addition will cause the total N_2O emissions to be the same in all aggregate sizes.

Soil pH, in the field trial, had a significant effect on total N_2O emissions from a grazed pasture soil. Therefore, the hypothesis that pH will significantly effect N_2O emissions is accepted. In agreement with Weslien et al. (2009), the field trial results detailed in Chapter 5, demonstrate that total N_2O emissions are significantly higher in the acidic pH soil compared

to the native and basic pH soil. The soil pH alters denitrification pathways, and in an acidic pH, although denitrification rates are lowered, the enzyme responsible for the conversion of N_2O to N_2 (N_2O-reductase) is inhibited (Wijler & Delwiche 1954; Weslien et al. 2009). In the field trial, it is hypothesised that the higher N_2O emissions in the acidic pH soil was caused by incomplete denitrification due to the inhibition of N_2O-reductase. However, in the basic pH, low N_2O emissions were caused by complete denitrification and the formation of N_2 (Stevens et al. 1998; Clough et al. 2004). The high N_2O emissions in the acidic pH soil and low N_2O emissions in the basic pH soil, demonstrate that with short term, artificial soil pH changes (1-1.5 pH units), N_2O emissions from an agricultural soil can be altered.

6.1.2 Ammonia oxidising communities

In the incubation trial, soil aggregate size had no effect on ammonia oxidising bacteria (AOB) or ammonia oxidising archaea (AOA) *amoA* gene abundance, and so the hypothesis that soil aggregate size would have a significant effect on ammonia oxidising communities must be rejected. With urine addition AOB *amoA* gene abundance increased significantly in all aggregate sizes. However AOA *amoA* gene abundance decreased with urine addition in the large aggregate size only. This is thought to be caused by the ability of large aggregates to hold more NO_3^- in their micropores (Diba et al. 2011) thus inhibiting the AOA abundance further.

In the field trial, the hypothesis that soil pH would have a significant effect on ammonia oxidising communities can be accepted. In the basic pH soil, with the addition of urine, AOB *amoA* gene abundance was significantly higher compared to the acidic pH soil. This is thought to be caused by the greater ammonia (NH_3) availability at a high pH (Dancer et al. 1973), due to the de-protonation of ammonium (NH_4^+) (Frijlink et al. 1992), and increased nitrification rates at a high soil pH (Morkved et al. 2007). The soil NH_4^+ and NO_3^- concentrations suggest this was occurring, as NH_4^+ concentrations were lowest in the basic pH soil, indicating that NH_3 may have been present instead, and NO_3^- concentrations were highest in the basic pH soil demonstrating greater NH_3 oxidation.

With urine addition, AOA *amoA* gene abundance was significantly higher in the acidic pH soil compared to the basic pH soil. AOA *amoA* gene abundance initially decreased with the addition of urine in the acidic pH, indicating inhibition (Di et al. 2010b). However, as the urine was hydrolysed, and the NH_4^+ oxidized, AOA *amoA* gene abundance continued to increase, with significantly higher AOA *amoA* gene abundance compared to the basic pH soil

at day 90. Therefore, the hypothesis that AOA will dominate at a low pH is accepted. This is similar to the findings of Nicol et al. (2008) who found as the pH decreased, AOA abundance increased. In addition, Zhang et al. (2011) and Gubry-Rangin et al. (2010) found that AOA dominate nitrification processes in acidic soils.

When comparing AOB and AOA *amoA* gene abundances, in the incubation trial, AOB *amoA* gene abundance was higher than AOA *amoA* gene abundance in all treatments. This was also found for Canterbury soils in Di et al. (2009). Similarly, in the field trial, AOB *amoA* gene abundances were higher than AOA *amoA* gene abundances in the native and basic pH soil in all treatments. However, in the acidic pH soil, AOA *amoA* gene abundance was higher than AOB *amoA* gene abundance in those treatments without urine amendment. This demonstrates that even with short term soil pH alteration, AOA communities will play an important role in the nitrification process in an acidic soil. However, when N contents are high AOB will dominate the nitrification process (Di et al. 2009), regardless of soil pH and aggregate size. Therefore supporting the idea that AOB prefer nitrogen rich environments whereas AOA prefer nitrogen poor, low pH environments (Di et al. 2009; Erguder et al. 2009; Di et al. 2010b).

In accordance with Di et al. (2009), the application of DCD, in the urine+DCD treatment, significantly reduced AOB *amoA* gene abundances in both the incubation and field trial, regardless of aggregate size and soil pH. The largest reduction was seen in the basic soil pH which is thought to be caused by a higher overall AOB *amoA* gene abundance in an alkaline pH soil. AOA abundances were unaffected by the addition of DCD in all aggregate sizes and in the native and basic soil pH which is in agreement with Di et al. (2009). However, in agreement with Zhang et al. (2011) AOA abundances decreased with DCD addition in the acidic pH soil. The addition of DCD without urine did not affect AOA abundances in the incubation and field trial, which suggests that urine rather than DCD inhibits AOA growth in the urine+DCD treatment.

6.1.3 DCD effectiveness

Regardless of soil aggregate size, on average DCD significantly reduced N_2O emissions from urine amended soils by 79%, and NO_3^- concentrations by 71%, allowing the hypothesis that DCD would remain effective at all soil aggregate sizes to be accepted.

In the field trial, DCD was more effective in the acidic pH soil, significantly reducing total N_2O emissions by 64% and NO_3^- concentrations by an average of 65%. In the basic and native pH soil, however, only peak N_2O emissions, at day 7 and 14, were significantly reduced with DCD addition. Furthermore, compared to the acidic pH soil, a lower reduction in NO_3^- concentrations in the native and basic pH soil was seen.

Decreased effectiveness in the higher soil pH treatments is thought to be caused complete denitrification producing a higher proportion of N_2 rather than N_2O. In addition, DCD was undetectable by day 85 in the native and basic pH soil, whereas in the acidic pH soil DCD remained in detectable amounts for the duration of the trial. The decrease in DCD longevity is thought to be caused by an increase in microbial activity in alkaline soils (Slangen & Kerkhoff 1984) which may have enhanced the microbial degradation of DCD (Puttanna et al. 1999b). This is supported by the AOB *amoA* gene abundance which was significantly higher in the basic pH soil. The results in Chapter 5 therefore demonstrate that DCD was less effective at reducing N_2O emissions and NO_3^- concentrations from a urine amended soil at a high soil pH which allows the hypothesis that DCD would be more effective at a low soil pH to be accepted.

In accordance with Kelliher et al. (2008) and Di and Cameron (2004), the DCD half-life in the incubation trial at 10˚C was 87 days. Comparatively, in the field trial the DCD half-life was low, with a half-life of only 14 days. However, the temperature in the field trial was warmer, with daily maximum temperatures averaging 14.9˚C. In addition, in the field trial, high rainfall occurred on day 1. This could have caused rapid movement of DCD out of the topsoil (0-7.5 cm) via macropore flow and out of the 'active' zone (Shepherd et al. 2012).

6.1.4 Implications for future farm management

The incubation trial used single aggregate sizes as treatments which is not realistic in a soil profile. Naturally, soils contain a log normal distribution of aggregate sizes (Smith 1980). However, mechanisms demonstrated in the incubation trial show that although initially larger aggregates may produce higher N_2O emissions, eventually due to small aggregate instability, the N_2O emissions will be the same across aggregate sizes. Furthermore the initial peak seen in the large and medium aggregates validates that anaerobic zones may be found in aggregates down to 4 mm (Sexstone et al. 1985). The ability of DCD to remain effective at all aggregate sizes demonstrates its usefulness as a mitigation tool in agricultural systems.

The field trial used artificial chemicals to alter the soil pH over a short term. Although this is not used in farm management, alteration of soil pH does occur due to fertiliser and lime addition. The results demonstrate that even a small decrease in pH (1-1.5 units) can cause a significant increase in total N_2O emissions. Thus, liming may be a mitigation option to decrease N_2O emissions from soils. However the implications for other greenhouse gas emissions from soil is unknown. In addition, the field trial validates the importance of DCD use, especially in acidic soils where its longevity and effectiveness at reducing N_2O emissions and NO_3^- concentrations is enhanced.

6.2 Future Research

In view of the results from the incubation trial, it is suggested that future research into the effects of aggregate size on N_2O emissions and microbial communities is undertaken incorporating a larger variation in aggregate sizes, greater than 2mm. This is because the medium (2-4 mm) and the large (4-5.6 mm) aggregates had similar N_2O fluxes.

It is hypothesized that aggregate instability is linked to the "switch" in N_2O emissions at day 66, where small aggregates amended with urine began to emit more N_2O than large and medium aggregates amended with urine. Measuring aggregate stability throughout the trial would help to support this hypothesis and the work done by Uchida et al. (2008). Furthermore, measuring organic carbon would identify whether the smaller aggregates released more labile carbon over time. In addition, although all aggregate sizes were packed to the same bulk density, a measurement of aeration would help to determine if there are differences in inter-aggregate pore space, as smaller aggregates may pack tighter than large aggregates.

In the field trial, it is hypothesised that the reason for higher N_2O emissions in the acidic pH soil was as a result of the inhibition of the N_2O-reductase and thus incomplete denitrification. Measuring the denitrifiers responsible for this enzyme would indicate whether this hypothesis is correct. Furthermore, measuring the emissions of N_2 would enable the ratio of N_2O:N_2 to be measured, and therefore it could be determined if complete denitrification was the reason for lower N_2O emissions in the basic pH soil.

In the incubation and field trial, measuring the abundance of the ammonia oxidising gene, *amoA*, provided an insight into the mechanisms involved in N_2O production. However assessing denitrifying communities would help link NO_3^- concentrations to N_2O emissions, especially in the incubation trial. Furthermore, continuing to extract DNA throughout the trial would help to explain latter N_2O and NO_3^- concentration trends. In the field trial, AOA *amoA* gene abundance increased in the acidic pH, similar to findings by Zhang et al. (2011). It would be interesting to identify if the activity of the AOA also increased in the acidic pH. This would identify if AOA did "take over" nitrification processes in the acidic soil or whether only the abundance increased.

DCD was effective at all aggregate sizes in the incubation trial; however DCD was more effective at a lower soil pH in the field trial. It would be interesting to identify the optimal pH

for DCD effectiveness and whether it continues to be more effective and have higher longevity at a soil pH less than 4.5.

In comparison to other published data (Di & Cameron 2004; Kelliher et al. 2008) and the incubation trial, the half-life of DCD in the field trial was reduced. It is assumed the low half-life was caused by a dilution effect caused by the high rainfall at day 1 and enhanced degradation by high temperatures during the trial period. Measuring the effectiveness of DCD at various soil pH in a controlled temperature and rainfall environment would identify whether it was the soil pH, high microbial abundances, or climatic factors which caused DCD's low half-life.

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Appendices

Appendix 1: Soil Characteristics

Table 1.1: Soil characteristics of the Temuka clay loam soil used in the incubation and field trial. Results from Analytical Research Laboratories.

Soil characteristic	Unit	Value
pH		5.78
Olsen P	µg/mL	17
CEC	me/100g	8.08
Magnesium	me/100g	1.89
Potassium	me/100g	1.60
Sodium	me/100g	0.19
Organic matter	% W/W	75.8
Total N	% W/W	0.34
Total C	% W/W	3.75
C:N ratio	ratio	11

Appendix 2: Incubation trial additional results

Table 2.1: Cumulative N_2O (g N_2O-N/ha) up to Day 51 and Day 288 for incubation study: main effect mean table.

	Day 51		Day 288	
	Log mean	(Back transform mean)	Log mean	(Back transform mean)
MAIN EFFECTS:				
Aggregate size				
Large	2.395	(248)	2.876	(752)
Medium	2.356	(227)	2.851	(710)
Small	2.239	(173)	2.765	(582)
LSD (5%)	0.098		0.067	
Significance of contrasts				
Linear trend p-value	0.003		0.040	
Urine				
0 urine-N kg/ha	1.727	(53)	2.371	(235)
700 urine-N kg/ha	2.933	(857)	3.264	(1836)
LSD (5%)	0.080		0.055	
Significance of difference				
(Nil versus urine) p-value	<0.001		<0.001	
DCD				
0 DCD kg/ha	2.415	(260)	2.989	(975)
10 DCD kg/ha	2.244	(175)	2.646	(443)
LSD (5%)	0.080		0.055	
Significance of difference				
(Nil versus DCD) p-value	<0.001		<0.001	

Table 2.2: Significant interaction tables for cumulative N_2O emissions (g N_2O-N/ha) up to Day 51 and Day 228 in incubation trial.

	Day 51		Day 288	
	Log_{10} mean	(Back transformed mean)	Log_{10} mean	(Back transformed mean)
(a) Aggregate size and Urine				
Large, 0 urine-N kg/ha	1.733	(54)	2.359	(229)
Medium, 0 urine-N kg/ha	1.719	(52)	2.418	(262)
Small, 0 urine-N kg/ha	1.728	(53)	2.334	(216)
Large, 700 urine-N kg/ha	3.058	(1142)	3.311	(2046)
Medium, 700 urine-N kg/ha	2.992	(981)	3.285	(1928)
Small, 700 urine-N kg/ha	2.749	(561)	3.196	(1570)
LSD (5%)	0.139		0.095	
Significance of interaction contrast				
Urine x Agg. Size (Linear trend) p-value	0.003		0.183	
(b) Aggregate size and DCD				
Large, 0 DCD kg/ha	2.456	(286)	2.968	(928)
Medium, 0 DCD kg/ha	2.436	(273)	3.017	(1040)
Small, 0 DCD kg/ha	2.354	(226)	2.980	(955)
Large, 10 DCD kg/ha	2.334	(216)	2.702	(504)
Medium, 10 DCD kg/ha	2.275	(188)	2.686	(485)
Small, 10 DCD kg/ha	2.123	(133)	2.550	(355)
LSD (5%)	0.139		0.095	
Significance of interaction contrast				
DCD x Agg. Size (Linear trend) p-value	0.265		0.019	
(c) Urine and DCD				
0 urine-N kg/ha, 0 DCD kg/ha	1.741	(55)	2.371	(235)
0 urine-N kg/ha, 10 DCD kg/ha	1.712	(51)	2.370	(234)
700 urine-N kg/ha, 0 DCD kg/ha	3.090	(1230)	3.606	(4036)
700 urine-N kg/ha, 10 DCD kg/ha	2.776	(597)	2.922	(982)
LSD (5%)	0.113		0.078	
Significance of interaction contrast				
Urine x DCD p-value	<0.001		<0.001	

Table 2.3: Main effects table for soil NO_3^- concentration (mg NO_3^--N/(g of soil)) at three sampling days during the incubation trial.

	Day 1		Day 84		Day 297	
	Log mean	(Back transform mean)	Log mean	(Back transform mean)	Log mean	(Back transform mean)
MAIN EFFECTS: **Aggregate size**						
Large	0.703	(5.05)	1.818	(65.8)	2.304	(201)
Medium	0.828	(6.73)	1.896	(78.7)	2.298	(198)
Small	0.984	(9.64)	1.884	(76.6)	2.264	(183)
LSD (5%)	0.073		0.046		0.043	
Significance of contrasts						
Linear trend p-value	<0.001		0.006		n.s	
Urine						
0 urine-N kg/ha	0.954	(8.99)	1.411	(25.8)	1.979	(95)
700 urine-N kg/ha	0.723	(5.28)	2.322	(210)	2.560	(363)
LSD (5%)	0.059		0.037		0.035	
Significance of difference						
(Nil vs. urine) p-value	<0.001		<0.001		<0.001	
DCD						
0 DCD kg/ha	0.834	(6.82)	2.016	(104)	2.412	(258)
10 DCD kg/ha	0.843	(6.97)	1.716	(52.0)	2.127	(114)
LSD (5%)	0.059		0.037		0.035	
Significance of difference						
(Nil vs. DCD) p-value	n.s		<0.001		<0.001	

Appendix 3: Field trial additional results

Table 3.1: Field trial pH values, treatment mean table for day 7 and day 56.

	Day 7			
Treatment	Control	DCD-only	Urine-only	Urine + DCD
Basic	7.03	6.94	7.14	7.34
Native	6.32	6.33	6.54	6.89
Acidic	5.14	5.30	6.41	6.29
LSD (5%)	0.33			
	Day 56			
Basic	6.98	7.33	7.20	7.04
Native	6.25	6.09	6.39	6.42
Acidic	4.44	4.33	4.95	5.35
LSD (5%)	0.27			

Table 3.2: Main effects table for cumlative N_2O emissions (g N_2O-N/ha) up to day 13 and 96.

	Day 13		**Day 96**	
MAIN EFFECTS: **Soil pH**	Log_{10} Mean	BT Mean	Log_{10} Mean	BT Mean
Acidic	2.038	109	2.773	593
Native	2.076	119	2.793	621
Basic	1.829	67	2.707	541
LSD (5%)	0.180		0.603	
Significance of contrasts				
Linear trend p-value	0.019	0.260	0.798	0.434
Urine				
0 urine-N kg/ha	1.380	24	2.455	285
700 urine-N kg/ha	2.614	411	3.077	1194
LSD (5%)	0.147		0.147	
Significance of difference				
(Nil vs. urine) p-value	<0.001		<0.001	
DCD				
0 DCD kg/ha	2.121	132	2.806	640
10 DCD kg/ha	1.872	74	2.709	533
LSD (5%)	0.147		0.147	
Significance of difference				
(Nil vs. DCD) p-value	<0.001		0.189	

Table 3.3: Main effects table for NO_3^- concentration (mg NO_3^--N/(g of soil)) at day 14 and 56.

	Day 14	Day 56
MAIN EFFECTS: **Soil pH**		
Acidic	6.9	4.67
Native	15.2	7.40
Basic	227.58.6	8.07
LSD (5%)	13.28	4.08
Significance of contrasts		
Linear trend p-value	0.012	0.21
Urine		
0 urine-N kg/ha	2.5	1.79
700 urine-N kg/ha	31.3	12.03
LSD (5%)	10.84	3.33
Significance of difference		
(Nil vs. urine) p-value	<0.001	<0.001
DCD		
0 DCD kg/ha	23.8	8.66
10 DCD kg/ha	9.3	4.77
LSD (5%)	10.84	3.33
Significance of difference		
(Nil vs. DCD) p-value	0.011	0.02

Table 3.4: Treatment mean table for NO_3^- concentration (mg NO_3^--N/(g dry soil)), day 14 and 56.

Day 14				
Treatment	Control	DCD-only	Urine-only	Urine + DCD
Acidic	0.40	0.80	19.50	6.80
Native	1.80	1.20	30.90	26.90
Basic	5.90	0.70	84.10	19.50
LSD (5%)	26.56			
Day 56				
Acidic	0.03	0.00	16.33	2.33
Native	1.66	2.73	14.41	10.82
Basic	1.78	2.20	17.73	10.56
LSD (5%)	8.16			

Table 3.5: Main effects table for soil ammonia concentration (mg NH_4^+-N/kg) at day 14 and day 56.

	Day 14	Day 56
Main effects: Soil pH		
Acidic	74.2	38.4
Native	36.5	5.6
Basic	39.2	4.3
LSD (5%)	22.5	16.3
Significance of contrasts		
Linear trend p-value	0.003	<0.001
Urine		
0 kg urine-N/ha	7.6	4.7
700 kg urine-N/ha	92.3	27.6
LSD (5%)	18.4	13.3
Significance of difference		
(nil vs. Urine) p-value	<0.001	0.001
DCD		
0 kg DCD/ha	52.7	8.9
10 kg DCD/ha	47.2	23.4
LSD (5%)	18.4	13.3
Significance of difference		
(nil vs. DCD) p-value	0.545	0.035

Table 3.6: Treatment mean table for soil ammonium concentration (NH_4^+-N/kg) at day 14 and day 56.

Day 14				
	Control	**DCD-only**	**Urine-only**	**Urine+DCD**
Acidic	5.2	5.9	164.1	121.4
Native	7.0	13.6	66.0	59.4
Basic	11.0	3.1	63.0	79.8
LSD (5%)	45.1			
Day 56				
Acidic	8.3	6.9	26.8	111.8
Native	3.5	3.8	7.2	8.0
Basic	3.3	2.2	4.4	7.5
LSD (5%)	32.7			

Table 3.7: Treatment means for AOA *amoA* gene abundance at day 90 of the field trial (BT = back transformed).

	Control		DCD-only		Urine-only		Urine+DCD	
	Log_{10} Mean	**BT Mean**	**Log_{10} Mean**	**BT Mean**	**Log_{10} Mean**	**BT Mean**	**Log_{10} Mean**	**BT Mean**
Acidic	7.414	2.59 $x10^7$	7.199	1.58 $x10^7$	7.555	3.60 $x10^7$	7.127	1.34 $x10^7$
Native	7.348	2.23 $x10^7$	7.562	3.65 $x10^7$	7.405	2.54 $x10^7$	7.330	2.14 $x10^7$
Basic	7.381	2.40 $x10^7$	7.384	2.24 $x10^7$	7.281	1.91 $x10^7$	7.322	2.15 $x10^7$
LSD (5%)	0.272							

Table 3.8: Main effects table for weighted averages AOB and AOA *amoA* gene abundance (BT = back transformed).

	AOB		AOA	
	Log_{10} Mean	**BT Mean**	**Log_{10} Mean**	**BT Mean**
Acidic	7.350	2.24 $x10^7$	7.421	2.64 $x10^7$
Native	7.440	2.75 $x10^7$	7.438	2.74 $x10^7$
Basic	7.602	4.00 $x10^7$	7.343	2.20 $x10^7$
Linear trend p-value	0.002		0.094	
LSD (5%)	0.135		0.091	
No Urine (0kg urine-N/ha)	7.342	2.20 $x10^7$	7.441	2.76 $x10^7$
Urine (700kg urine-N/ha)	7.585	3.85 $x10^7$	7.360	2.29 $x10^7$
Urine vs. Nil (p-value)	<0.001		0.033	
LSD (5%)	0.110		0.074	
No DCD (0 kg DCD/ha)	7.539	3.46 $x10^7$	7.406	2.55 $x10^7$
DCD (10 kg DCD/ha)	7.388	2.44 $x10^7$	7.395	2.48 $x10^7$
DCD vs. Nil (p-value)	0.009		0.782	
LSD (5%)	0.110		0.074	

Table 3.9: Treatment mean table for weighted average AOB *amoA* gene abundance (BT = back transformed).

	Control		DCD only		Urine only		Urine+DCD	
	Log_{10} Mean	**BT Mean**	**Log_{10} Mean**	**BT Mean**	**Log_{10} Mean**	**BT Mean**	**Log_{10} Mean**	**BT Mean**
Acidic	7.142	1.39×10^7	7.251	1.78×10^7	7.542	3.48×10^7	7.464	2.91×10^7
Native	7.486	3.06×10^7	7.321	2.09×10^7	7.717	5.21×10^7	7.233	1.71×10^7
Basic	7.483	3.04×10^7	7.368	2.33×10^7	7.864	7.31×10^7	7.692	4.92×10^7
LSD (5%)	**0.270**							

Table 3.10: Treatment mean table for weighted average AOA *amoA* gene abundance (BT = back transformed).

	Control		DCD only		Urine only		Urine+DCD	
	Log_{10} Mean	**BT Mean**	**Log_{10} Mean**	**BT Mean**	**Log_{10} Mean**	**BT Mean**	**Log_{10} Mean**	**BT Mean**
Acidic	7.448	2.81×10^7	7.468	2.94×10^7	7.510	3.24×10^7	7.258	1.81×10^7
Native	7.430	2.69×10^7	7.548	3.53×10^7	7.405	2.54×10^7	7.368	2.33×10^7
Basic	7.329	2.13×10^7	7.425	2.66×10^7	7.313	2.06×10^7	7.305	2.02×10^7
LSD (5%)	**0.182**							